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TITLE: Prevention of Ovarian High-Grade Serous Carcinoma by Elucidating Its Early Change•

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14. ABSTRACT: Project 1 We will determine the early molecular changes in STIC and their biological significance in developing high-grade serous carcinoma. marker selection and sample preparation will begin in the next coming months. Project 2 We will evaluate whether the presence of a STIC is associated with different clinical manifestations and/or outcome compare to those patients in whom a STIC was not identified. Molecular profiling will be initiated after quality control checking. Project 3 We will identify the early molecular changes that precede the development of STICs using gene expression analysis of morphologically normal FTE from high-risk women compared to FTE from normal control specimens and use an <i>in vitro</i> system and a mouse model to generate a molecularly defined carcinoma resembling HGSC from FTE and OSE using oncogenes expressed in ovarian carcinoma. Project 4 We plan to if the statin drugs are effective in preventing STIC formation and suppress tumor progression in the OVGp1 mouse model that spontaneously develops STIC and neoplasms.. Project 5 With the data and cases piling up, we will be able to address the molecular and epidemiologic profile of putative precursor lesions including STIC in the fallopian tubes and ovaries from women at high-risk for ovarian cancer. Also, a pilot study will be performed to determine the most cost-effective way to prepare the tissue sections for studies related to study early tumor development in ovarian cancer. This information will be shared with science community.					
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Section I- Purpose and Scope of the Research Effort (all projects)

The purpose of this Ovarian Cancer Consortium is to test the overarching hypothesis that serous tubal intraepithelial carcinoma (STIC) is the precursor and not a metastasis of many, if not most, pelvic high-grade serous carcinomas (HGSCs) but we believe all the other proposed candidates should be investigated in order to determine if STIC is the precursor of all ovarian and pelvic HGSCs or that OSE and CICs harbor precursor lesions as well. Our objective is to then carefully characterize the morphologic, molecular genetic, immunohistochemical (IHC) and epidemiologic features of the precursor lesions(s) (Projects 1-5). If STIC is shown to be the precursor lesion, the data generated by our studies will provide the rationale for our long-term objective, which is the prevention of ovarian HGSC by surgical or medical approaches. Opportunities in the field of cancer prevention have never been greater and therefore our Consortium will undertake innovative studies aimed at providing the scientific underpinning for reducing the burden of ovarian cancer through prevention. Finally, it is important to note that clear cell, endometrioid and mucinous carcinomas are clinically important but they represent only 25 % of all ovarian carcinomas and account for 10% of deaths. In contrast, as noted above, HGSC represents 75% of all ovarian cancers and accounts for 90% of the deaths. Accordingly, we will focus our studies exclusively on the early events associated with HGSC, as it clearly is the most important histologic subtype in terms of frequency and mortality.

The main research efforts in this Consortium are summarized in our five projects.

Project 1: Evaluate whether STICs are precursor lesions and not metastases from a primary ovarian HGSC by analyzing STICs from women with concomitant ovarian HGSCs and determining if the ovarian tumors have acquired additional molecular alterations compared to the STICs which would confirm that STICs are precursor lesions.

Project 2: Evaluate all the proposed site of origin (FTE, OSE, CICs and peritoneum) showing that the morphologic and molecular features of tubal, ovarian and primary peritoneal HGSCs are the same and in conjunction with Project 1 confirming our hypothesis that many, if not most, HGSCs originate in the fimbria and involve the ovary secondarily.

Project 3: Identify the early molecular changes that precede the development of STICs using gene expression analysis of morphologically normal FTE from high-risk women compared to FTE from normal control specimens and use an in vitro system and a mouse model to generate a molecularly defined carcinoma resembling HGSC from FTE and OSE using oncogenes expressed in ovarian carcinoma.

Project 4: Locate and characterize precursor lesions of “ovarian” cancer in a mouse model and explore the role of ovulation and changes in the microenvironment of the ovary and tube in “ovarian” carcinogenesis using human tubal xenografts in nude mice.

Project 5: Determine the molecular and epidemiologic profile of putative precursor lesions in the fallopian tubes and ovaries from women at high-risk for ovarian cancer. In addition, Project 5 will determine if these biomarkers and associated precursor lesions are modifiable by oral contraceptives (OCPs) or anti-inflammatory agents as OCPs in particular are known to prevent ovarian cancer and impact survival.

Section II, III and IV (5 projects are reported here individually)

Project 1: Evaluate whether STICs are precursor lesions and not metastases from a primary ovarian HGSC by analyzing STICs from women with concomitant ovarian HGSCs and determining if the ovarian tumors have acquired additional molecular alterations compared to the STICs which would confirm that STICs are precursor lesions.

Research site: Johns Hopkins University

Project Leader: Ie-Ming Shih

Co-investigators: Doug Levine (MSKCC), Robert J. Kurman (JHU)

Section II. Progress to Date

Task 1. Determine the clonal relationship and tumor progression pathway from STIC to invasive high-grade serous carcinoma.

Task 1a. Case selection and sample preparation including LCM, DNA extraction (1-20 months)

Progress: The Pathology Core has made progress in collecting cases for the studies proposed in Project 1 is requesting the new STIC cases from the Pathology Core to perform this study. Previously we have collected STIC cases studies (1-4), and now we are collecting new cases from different research sites. The actual number of STICs and associated HGSCs will be known and reported after Pathology Core has compiled all the cases and after centralized pathology review.

Task 1b. TP53 mutational analysis of all the potential precursor lesions of HGSC (8-24 months).

Progress: The task has been performed and the results have been described in the last progress report and, most importantly, the data have been recently published in J Pathol, 226:421-426, 2012. PMID:21990067. So we will not reiterate the progress here.

Task 1c. Allelic imbalance assay by digital SNP analysis and data analysis (24-36 months)

Progress: This part of progress is new to this report and represents the main progress that has been made in the past 6 months. We have collected formalin-fixed paraffin-embedded (FFPE) samples from five patients, each sample consisting of pelvic high-grade serous carcinoma (HGSC), normal tissue (uterus), and serous tubal intraepithelial carcinoma (STIC). In two of the five patients, we acquired HGSC from multiple regions: three regions (bilateral ovaries and omentum) in one patient and two regions (ovary and omentum) in another. Genomic DNA was purified from 8 HGSC and 5 normal specimens and enriched for coding regions (20,766 coding genes, 50Mbp) using in solution DNA capture approach. Enriched HGSC and normal DNA was sequenced using massively parallel sequencing instruments (100bp paired-end). In overall, we identified 56 ~ 127 (mean 84.5) somatic non-silent mutations in each HGSC. This successful result will pave the way for the next phase study by sequencing candidate genes in the corresponding STICs.

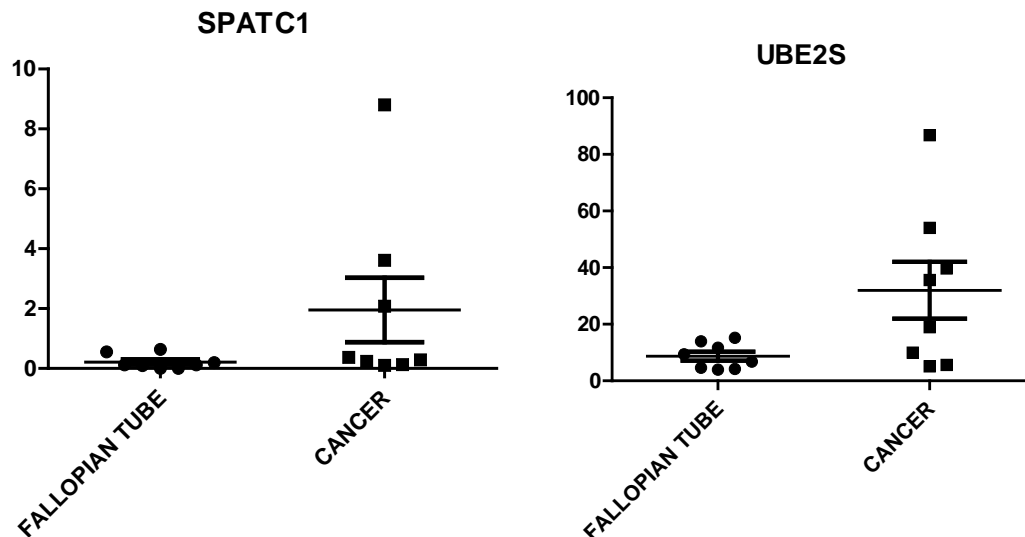
Task 2. Determine the early molecular changes associated with serous tubal intraepithelial lesions.

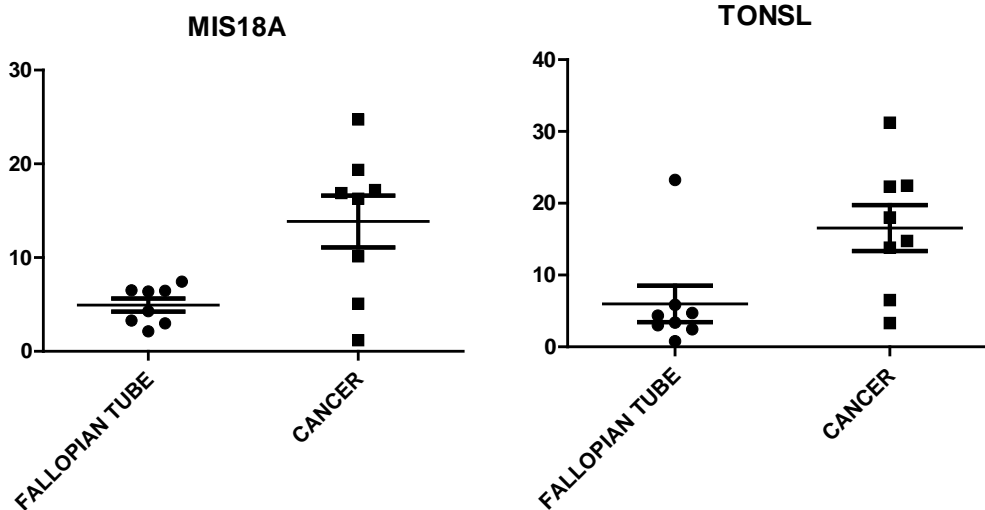
Task 2a. Immunohistochemistry study on ovarian cancer-associated markers on STICs and other putative precursor lesions (18-40 months)

Progress: As discussed in the previous report, we have finished a study by analyzing ALDH1A1 in normal fallopian tube, STIC and high-grade serous carcinoma (HGSC). In the last couple of months, we have revised the manuscript and resubmit our paper. This paper is now published in Modern Pathology (PMID: 25216223), the official journal of United States of American and Canadian Association of Pathologists (USCAP) with acknowledgement of this funding mechanism. Another research front we have accomplished is the transcriptome analysis of fallopian tube epithelium and high-grade serous carcinomas. As discussed in the previous report, we have recently initiated this project to identify the ovarian cancer-associated markers that can be studied in STICs. This is a highly significant study as there are no other similar studies reported, to our best knowledge, in the literature by comparing the fallopian tube epithelium and HGSC although there are numerous studies reporting the genes differentially expressed in HGSC as compared to ovarian surface epithelium (OSE). TCGA has

attempted to conduct a similar approach but they analyzed the whole fallopian tube rather than its epithelium. Since OSE is unlikely the cell origin of HGSC, our results should be fundamentally important in study HGSC and its precursor, STIC. In the past 6 months, we have finished the analysis and the main findings are summarized below.

We have performed RNAseq in directly lysed normal fallopian tube epithelial cells from 8 fresh normal fallopian tubes and compared its transcriptome to 8 HGSCs. The Agilent 2100 Bioanalyzer was used to check RNA integrity. 4ug of total RNA was processed using the mRNA-Seq sample prep kit following Illumina's protocol (Illumina San Diego, CA Cat# RS-100-0801) where mRNA was first isolated, fragmented and then used as a template for cDNA. The cDNA was amplified and enriched for 250 bp products. The library was further processed according to the manufacturer's protocol using both the Illumina Single Read Cluster Generation kit (GD-103-4001) to generate clusters on a 8 row flow cell and the 36 Cycle Sequencing kit V4 (FC-104-4002) to perform 42 cycles of sequencing on the Genome Analyzer (GA-II). Sequencing images were generated and base calls made utilizing Illumina's Analysis Pipeline software package. Hierarchical clustering using both CASAVA and CUFFLINKS separated fallopian tube epithelium samples from high-grade serous carcinoma samples. We found that the most significantly upregulated tumor-associated genes in high-grade serous carcinomas include Notch3, SMAD6, IGFBP3, BMP7, TGFBI, SPACT1, UBE2S, MIS18a, TONSL and LAMC1. The significant downregulated genes include OVGP1, ALDH1A1, ALDH1A2, WDR, TFF3 and DNAJA4. Among them, recent immunohistochemistry studies have shown that LAMC1 is upregulated in high-grade serous carcinoma while ALDH1A1 and OVGP1 are downregulated as compared to normal fallopian tube epithelium, indicating the robustness of our approach in identifying genes that are preferentially expressed in high-grade serous carcinomas and normal fallopian tube epithelium. We have recently published a paper describing the findings of ALDH1A1 (PMID: 25216223) acknowledging the DOD support. Representative data are shown in the figure panel below (y-axis is the relative expression levels). Taken together, our initial analysis of this study reveals highly interesting results in identifying differentially regulated genes in HGSC as compared to fallopian tube epithelial cells. It is expected that our results and database to be presented in the future will have implications for the future studies of ovarian HGSC and STIC.





Task 2b. In situ hybridization and/or mRNA expression analysis on those markers that the antibodies are not available (30-40 months)

Progress: So far, we have been fortunate to identify good antibodies (laminin C1, ALDH1A1, p53, topoisomerase II, cyclin E1, etc.) for immunostaining purposes and there is no need for us to consider *in situ* hybridization at this moment.

Task 2c. Verification of new markers from Project 3 in precursor lesions (24-56 months)

Progress: We have verified several new markers in STICs including LAMC1, ALDH1A1 and cyclin E1. The results have been reported in the past progress reports and will not be reiterated here. They have been or will be published in the following journals including J Pathol (PMID: 23378270), Am J Surg Pathol (PMID: 22892598) and Mod Pathology (PMID: 25216223).

Task 2d. Telomere FISH on STICs and other precursor lesions (36-48 months)

Progress: This part of study has been largely completed.

Task 2e. Data analysis and preparation for publications (40-60 months)

Progress: We are in the process of generating data although we continue publishing our results.

Section III. Problem Areas for Project 1

The problem area as mentioned in the previous progress report has now been solved and progress has been made. In brief, we will consider use an approach that is highly feasible to address this question related to Task 1. This approach is to perform whole exome sequence of HGSC and its normal tissues to identify somatic mutations. Then we will perform laser capture micro-dissection on STIC and analyze the mutations status on those genes that we identify in HGSC from the same cases. If we identify somatic mutations in HGSC but not in STIC, we will conclude that during tumor progression, HGSC acquires new mutations that are absent in STIC, a result supporting that STIC is the precursor lesion of HGSC. We plan to collect those eligible cases and access the cost related to this part of study in Task 1.

Section IV. Future Works in Project 1

Task 1C. TP53 mutational analysis of all the potential precursor lesions of HGSC.

We will prepare the gDNA from the STIC lesions and perform sequencing analysis to determine if the mutations indented in corresponding HGSCs can be detected. The results will be important to support or refute our working hypothesis if STIC is the precursor lesions of HGSC of the ovary.

Task 2a. Immunohistochemistry study on ovarian cancer-associated markers on STICs and other putative precursor lesions.

In the next 6 months, we will focus on validating the RNAseq data and identify the differentially expression genes in HGSC, so we can use those prioritized markers to validate in a panel of HGSCs in tissue microarrays and a cohort of STICs.

New DoD Consortium supported publications for Project 1:

Zeppernick F, Meinhold-Heerlein I, Shih IM. Precursors of ovarian cancer in the tube- STIC an update. J Ob Gyn Res, Oct 20. doi: 10.1111/jog.12550. [Epub ahead of print]. PMID: 253308222.

Chui MH, Wang Y, Wu RC, Seidman J, Kurman RJ, Wang TL, Shih IM. Loss of ALDH1A1 expression is an early event in the pathogenesis of ovarian high-grade serous carcinoma. Mod Pathol, Sep 12. doi: 10.1038/modpathol.2014.89. [Epub ahead of print]. PMID: 25216223

Ardighieri L, Lonardi S, Moratto D, Facchetti F, Shih IM, Vermi W, Kurman RJ. Characterization of the immune cell repertoire in the normal fallopian tube- implications for understanding ovarian carcinogenesis. Int J Gyn Cancer, 33:581-591, 2014. PMID: 25172297

Project 2: The relationship between serous tubal intraepithelial carcinoma and invasive pelvic serous carcinoma

INTRODUCTION:

Ovarian cancer has traditionally been thought to develop from the OSE or cortical inclusion cysts, but recent data suggest that a majority of advanced HGSC may originate from the fallopian tube epithelium. Although highly provocative, this hypothesis requires further validation and therefore we propose to analyze a large group of pelvic, which includes ovarian, tubal and primary peritoneal, HGSCs diagnosed using traditional criteria with and without STICs in women whose fallopian tubes have been processed using the SEE-FIM technique, currently the most comprehensive method of evaluating fallopian tube epithelium. Our primary objective is to determine whether there are subsets of HGSC, which have different molecular profiles and different clinical behavior based on their presumed site of origin or whether there are no differences and that they are essentially the same irrespective of their site of origin. We will also compare the molecular profiles of normal tissues to HGSCs as a whole and HGSCs with and without STICs placing specific emphasis on the ovarian surface and distal fallopian tube epithelium.

SUMMARY: Since the last interim report, significant progress has been made in Task 3c and 3d as outlined below. Specifically and briefly, we have processed all tumor samples for all genomic platforms.

BODY:

Task 1: Determine the frequency of STICs in patients with advanced pelvic HGSC.

Task 1a. IRB documents filed at each consortium sites. (Complete)

IRB documents have been completed by each site in the consortium.

Task 1b. Case identification. (Complete)

We have identified 228 cases of HGSC from MSKCC since 2009 when the SEE-FIM protocol was initiated. We have developed a data dictionary and data collection form for other consortium centers to contribute clinical data regarding STIC cases. Each center has agreed to provide additional clinical data to develop a more robust dataset.

Task 1c. Slides review and section recut (Complete).

Of the 228 cases identified from institutional databases, 18 cases were unavailable and 33 cases had insufficient tissue sections available to determine that a STIC lesion could not have been present. Of the remaining 177 cases, 104 are confirmed to have STIC lesions and 73 do not have STIC lesions based on comprehensive review of fully submitted fallopian tubes.

Task 1d. Tabulate frequency of STIC (Complete).

Progress: The frequency of fallopian tube cancer in this population is 59% and is much higher than early historic populations supporting the increase in diagnosis of tubal carcinoma with the advent of comprehensive tubal analysis.

Task 2: Evaluate whether the presence of a STIC is associated with different clinical manifestations and /or outcome compare to those patients in whom a STIC was not identified.

Task 2a: Collect clinical data from specimens identified in Task 1a, above. (Complete)

Clinical data has been collected from all MSKCC patients. Of the 215 patients identified, the median age was 62 years old; identical to population based reports of HGSC confirming the fact that our study population reflects the general population of HGSC patients. All patients had high-grade (grade 3) serous carcinoma with stage IIIC (N=159, 74%) or IV (N=56, 26%) disease. Optimal surgical cytoreduction was achieved in 194 patients (90.2%). All patients received platinum-based adjuvant chemotherapy after surgery. With relatively short median follow-up of 23 months, 12% of the patients have died and the median overall survival has not yet been reached.

Task 2b: Analyze clinical variables in the context of specimens with or without STIC

The presence or absences of STIC lesions has been confirmed in all cases from MSKCC that will qualify (sufficient tubal representation in pathology specimens). We will now be able to finalize our analysis of the clinical data in the context of STIC lesions for MSK cases. Additional analyses are ongoing based on further collection of cases from other consortium sites. We had been occupied with Task 3 during the last reporting period and will complete this task in the upcoming year.

Task 2c: Write manuscript and publish results

Pending. We will await data from other consortium institutions and then incorporate these findings into Task 2b.

Task 3: Compare the molecular features of advanced pelvic HGSCs with and without associated STIC to various normal pelvic tissues.

Task 3a. Collect 100 HGSC specimens that had SEE-FIM processing from Consortium sites; 10-20 specimens per site with the balance contributed from MSKCC (Complete)

We have identified 153 advanced ovarian cases across the consortium and have extracted nucleic acids from 102 cases with and without STIC lesions that yielded high quality nucleic acids. (50 STIC cases and 52 non-STIC cases, See Fig 1). We have received 29 qualifying HGSC cases from other consortium centers that have had comprehensive evaluation of the fallopian tubes as shown in Table 1. We were not able to receive any specimens from Yale as they do not have access to any retrospectively banked specimens. We have discussed the design issues again with Dr. Cope (Biostatistics core) and will run tumors and normal samples each as a single but separate batch.

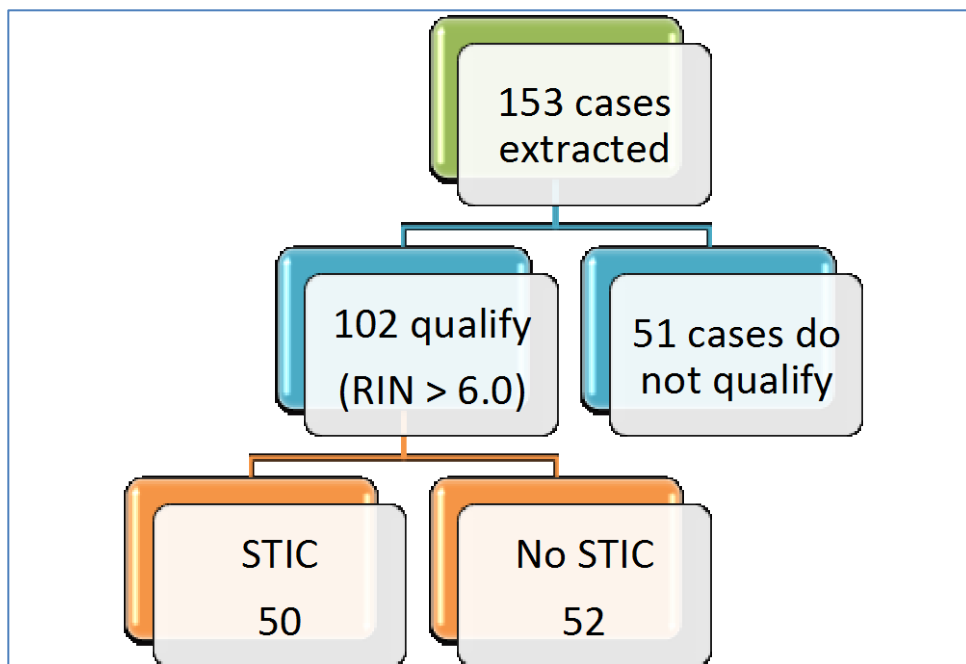


Figure 1. Consort diagram of case qualification for Task 3a.

	STIC	No-STIC	Total
MSKCC	44	29	73
Hopkins	3	10	13
Toronto	5	11	16
All institutions	52	50	102

Task 3b. Collect 10 normal tissues from each of 5 anatomic sites; 50 total tissues. Tissues to be collected across Consortium sites, with each site contributing at least 2 of each normal tissue type with the balance contributed from MSKCC (Complete)

To begin to address this task, we have optimized the methods for collecting normal tissue (SOP appended below). We have confirmed by cytology that these samples contain a predominance of epithelial or mesothelial cells, as appropriate. We are also able to recover 50-300 ng of DNA or RNA per sample. Approximately 50% of collected specimens yield good quality RNA (Fig 2.). We have collaborated with Dr. Tanner from Hopkins to collect normal samples. A MSK lab member travelled to Hopkins to train Dr. Tanner in collection technique. We have received one sample to date that failed QC. We have completed collection of normal tissue samples. Some samples have lower than desired RNA quality, but we have new approaches to recover these samples and generate reliable data. We will have at least 10 tissue samples from each normal tissue site to process.

RIN	Tube	Peritoneum	Ovary
1.0-3.9	14	21	9
4.0-4.9	5	5	2
5.0-5.9	4	2	3
6.0-8.0	4	4	14

Task 3c. Process all specimens on various genomic platforms

We now have a collection of 100 qualified samples. We have extracted nucleic acids for all qualified specimens. We will run them all in a single batch. We had planned on using various array platforms for genomic analyses. Since the proposal has been submitted most genomic studies of this scale have moved toward more modern methods of measurement. For this reason, we will measure gene expression with RNA sequencing on an Illumina HiSeq machine instead of using the Affymetrix U133A 2.0 transcription array. The RNAseq provide better coverage and direct, rather than indirect, assessment of gene expression. We will use the Affymetrix 6.0 SNP array instead of the oligonucleotide Agilent 1M CGH array for somatic copy number alterations, due to familiarity and reliable performance of the Affymetrix array. We will measure microRNA expression with the Nanostring platform as it also provides better coverage and direct, rather than indirect, assessment of gene expression when compared with the Agilent Human miRNA Microarray. Sufficient nucleic acids will be stored for technical validation procedures.

The Affymetrix 6.0 SNP array and the microRNA expression on the Nanostring platform have been completed for 96 samples. All samples passed secondary QC for both platforms. The data has been transferred to the biostatistical core at JHU for further analyses.

The RNAseq had been minimally problematic. Of the submitted 96 samples, 51 have passed QC and RNAseq data with 20-30M reads has been generated. This data will be sent to JHU for analyses in the next month. Of the 45 samples that failed initial QC, 31 had been replaced with new samples from the same patients (different part of the biospecimen). These new samples had passed initial preprocessing QC, but failed library preparation. After a total of 10 months of troubleshooting, the problem appeared to be with the ribosome depletion step. By adjusting the ribosome depletion reaction, all samples could be salvaged and RNA sequence generated. At this point, all genomic data has been generated for the tumor samples. We will begin to generate data for the normal samples during the upcoming year.

Task 3d. Analyze data on each platform according to proposal.

A preliminary analysis of the 96 samples suggests that there is no general distinction based on the presence or absence of STIC lesions. Figure 2 below show a preliminary analysis of the RNA sequencing data. No batch effects were identified either. A supervised analysis using known protein-coding genes with each gene expressed in at least 50% of samples, the 50% most variable genes were identified. Of the 9,236 most variable genes, 69 were differentially expressed at $P < 0.01$, none of which passed multiple comparison correction with stringent Bonferroni correction. This was less than expected by chance alone (92). Family-wise correction for multiple hypothesis testing (B-H) is pending. Further analyses to incorporate clinical parameters such as residual disease, platinum resistance, and survival outcome as positive controls is also pending.

Figure 3 below show a preliminary analysis of the microRNA data. No batch effects were identified. The dendrogram from the unsupervised analysis suggests segregation between STIC and No STIC cases, but there does not appear to be any structure to the data when visualized in the heatmap. A supervised analysis of the microRNA expression data using 220 of the most variable microRNAs, identified 14 differentially expressed genes at an FDR of < 10%. These potential true discoveries will need to be validated and further analyzed.

Figure 4 below show a preliminary analysis of the copy number alterations. There does not seem to be any differences between STIC and No STIC cases. Specifically each group has approximately 110 focal alterations, nearly evenly split between amplifications and deletions. These findings are consistent with our reports in the TCGA ovarian study. Most of the highly significant focal alterations are shared between groups.

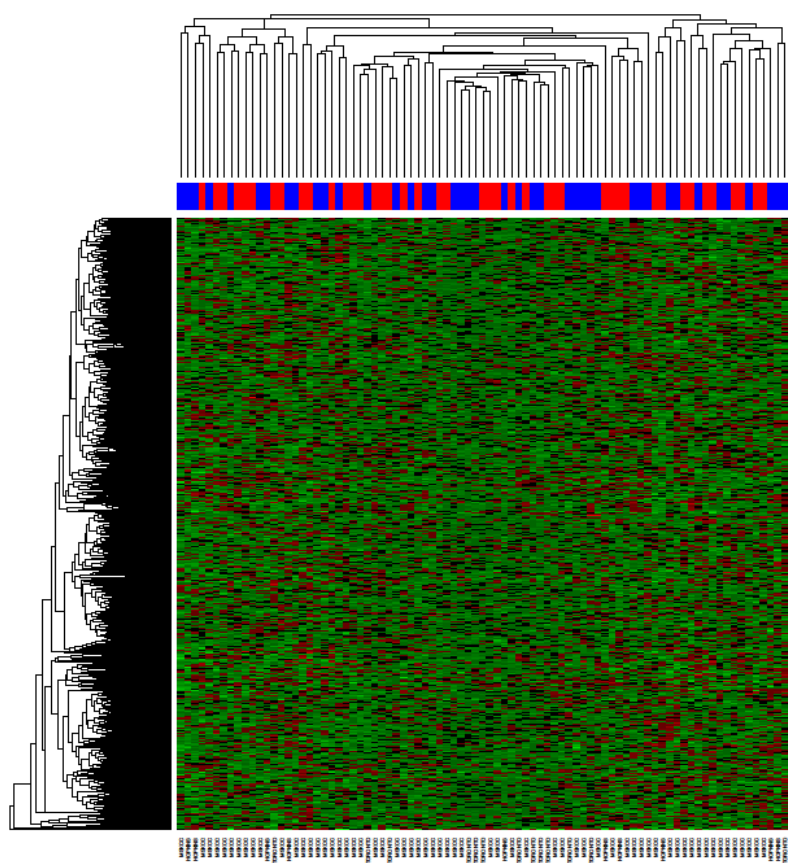


Figure 2. Unsupervised hierarchical clustering of RNA sequencing shows no clear separation between tumors with and without STIC lesions. Blue – STIC lesion present; Red – STIC lesion absent.

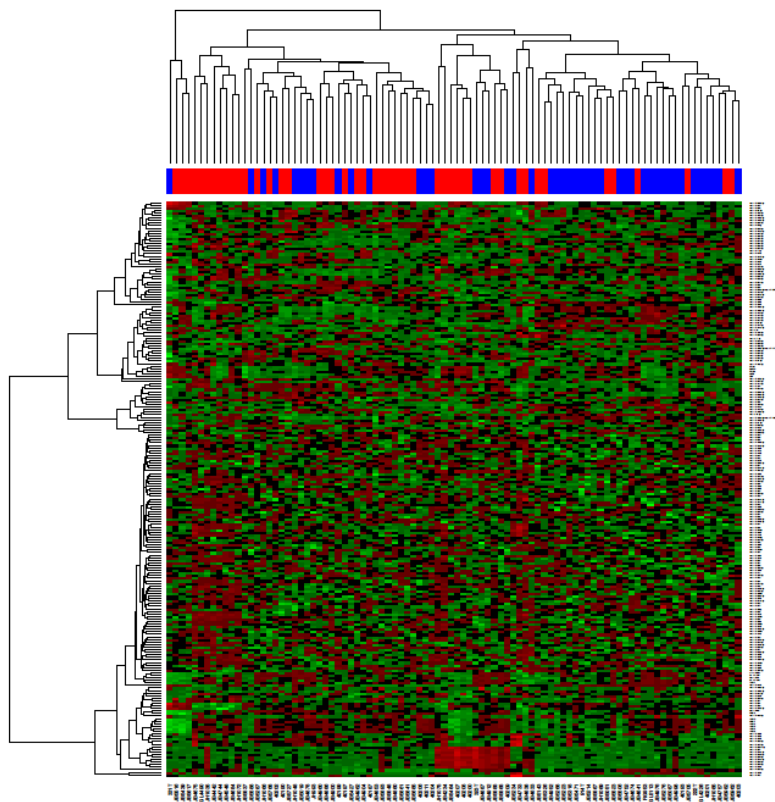


Figure 3. Unsupervised hierarchical clustering of microRNA expression shows no clear separation between tumors with and without STIC lesions. Blue – STIC lesion present; Red – STIC lesion absent.

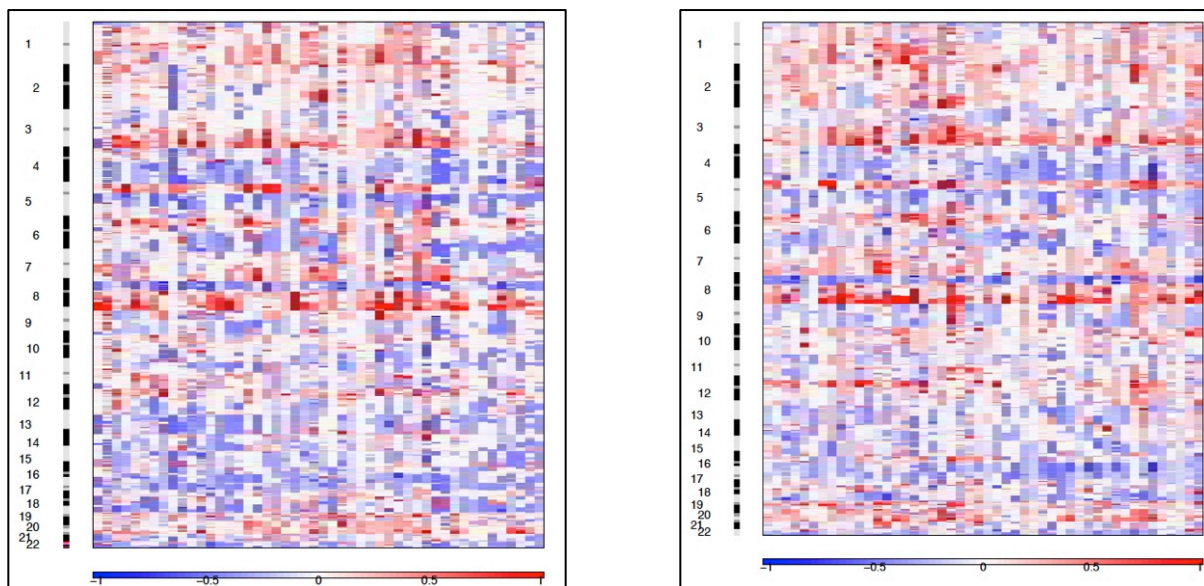


Figure 4. Segmental analysis of copy number alterations shows no clear separation between tumors with (left) and without STIC lesions (right).

Task 3e. Write manuscript and publish results

Pending

KEY RESEARCH ACCOMPLISHMENTS:

- We have generated all genomic data from tumor specimens
- We have collected all normal specimens needed for analysis
- Preliminary analysis demonstrated minimal differences between study groups.

REPORTABLE OUTCOMES:

An abstract from Task 3 has been accepted for poster presentation at the Annual Meeting of the Society of Gynecologic Oncologists in March 2015.

CONCLUSION:

To date we have found no significant genomic differences between HGSCs with or without precursor STIC lesions suggesting a common site of origin in the distal fallopian tube. This will lead to changes in our approach to ovarian cancer prevention and early detection.

REFERENCES: None.

APPENDICES: None.

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers. These have been included in the text above.

PROJECT 3. Identification of molecular changes preceding STICs in FTE from high-risk women using in vitro and in vivo models

Investigators: PI - Shaw, Shih

Research site: University of Toronto

INTRODUCTION:

In this project we will determine if the expression profiles of anatomically high risk FTE (fimbrial) from women at high genetic risk (BRCA1 mutation carriers) differs from the FTE profiles from women at low risk of HGSC, and we propose that these changes may play key roles in the earliest events of serous carcinogenesis. To this end, we will use a molecularly defined system to sequentially express ovarian cancer-associated genes including those identified in this project into ovarian surface epithelium (OSE) as well as fimbrial FTE to determine a) if FTE is more prone to neoplastic transformation and b) if the FTE-derived tumors more closely simulate HGSC than OSE-derived tumors.

Recently described precursors of High Grade Serous Carcinoma, the p53 signature, a latent precursor, and Serous Tubal Intraepithelial Carcinoma, a pre-malignant precursor, occur most frequently at the distal and fimbriated end of the fallopian tube (FTE). We recently demonstrated that the FTE of BRCA1 mutation carriers, at genetic risk of HGSC, have altered signaling pathways compared to controls. A key question is whether the gene expression differences identified at the ampulla between BRCA1 and non- mutation carriers is similar to differences at the fimbria. This study determines the transcriptome profiles of normal fimbrial FTE and normal ampulla FTE which may lead to insight of why the distal end of the fallopian tube is preferentially predisposed to malignant transformation

Specific Aim 1. Detect and select genes differentially expressed in morphologically normal fimbrial FTE from women at high genetic risk of HGSC.

Specific Aim 2. Model alterations associated with normal FTE from high-risk women and STIC in vitro and in vivo.

BODY:

1. Establish expression profiles of fallopian tube epithelium from BRCA1 mutation carriers and controls, and of serous cancers in mutation carriers.

Progress: To date, we have collected and processed over 200 formalin-fixed and/or cryopreserved cases of fallopian tube and fimbriae specimens. These cases include samples from BRCA1 and BRCA2 mutation carriers undergoing prophylactic surgery, patients undergoing debulking surgery for High Grade Serous Carcinoma, and patients undergoing salpingo-oophorectomy for non-malignant reasons. An integral aspect of Project 3 *Specific Aim 1* is to determine the relationship between hormonal response and BRCA mutation status in the normal fallopian tube epithelium. As a result, a significant effort has been placed on determining the menstrual status of samples collected – this included reviewing the endometrium of corresponding samples when available. We have completed histological reviews of fallopian tubes from BRCA1 and BRCA2 mutation carriers, along with matching controls and cancers and identified 84 cases that can be used for gene expression profiling. One of the key questions within this aim is whether the gene expression differences identified at the ampulla between BRCA1 and non-BRCA1 mutation carriers is similar to differences at the distal end of the fallopian tube – the fimbria. We have determined therefore, to answer the ampulla versus fimbria conundrum, it would be technically robust to use cryopreserved ampulla and fimbria from non-BRCA mutation carriers with known ovulation cycle status.

A). Micro-dissection of selected cryopreserved tissue samples

Using gene level differential expression analysis with the Affymetrix Expression Console software, we performed unsupervised hierarchical clustering analysis with all 24 samples. We used a fold change of < -2 or > 2 and ANOVA p-value < 0.05 as a cut-off criteria for selecting genes. The cases clustered predominantly by ovarian cycle status rather than by their differences in anatomical origin or their matched pair. There were 427 genes differentially expressed amongst the 4 groups – Fim-Luteal, Fim-Follicular, Amp-Luteal and Amp-Follicular. Independent of ovarian cycle status, very few differences (35 genes – SALL1, SERPINA3, ANXA13, PDK4, ME1, GSTA1, GSTA2 – genes involved in metabolic pathways) were observed between the ampulla and fimbria FTE (**Figure 1 and Figure2**). The epithelia of the anatomically high-risk fallopian tube – the fimbria, show few differences in gene expression profiles compared to the lower risk portion – the ampulla. Expression differences predominantly are in response to the hormonal milieu, i.e. the secretory and proliferative phases of the ovarian cycle. The increased anatomic risk of the fimbria is likely due to effects of the microenvironment, such as repeated exposure to follicular fluid at ovulation, rather than intrinsic differences of the FTE in the two sites.

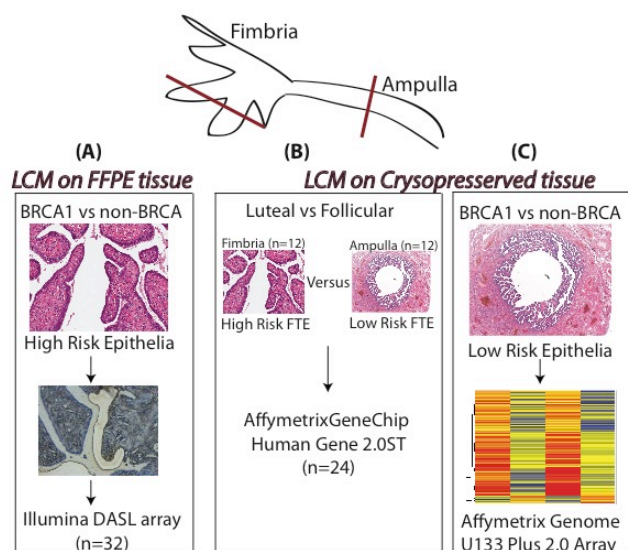


Figure 1. One of the key questions within this aim is whether the gene expression differences identified at the ampulla between BRCA1 and non-BRCA1 mutation carriers is similar to differences at the distal end of the fallopian tube – the fimbria. We have determined therefore, to answer the ampulla versus fimbria conundrum, it would be technically robust to use formalin-fixed paraffin embedded tissue fimbria from BRCA mutation carriers with known ovulation cycle status, using techniques our laboratory has used successfully in previous studies.

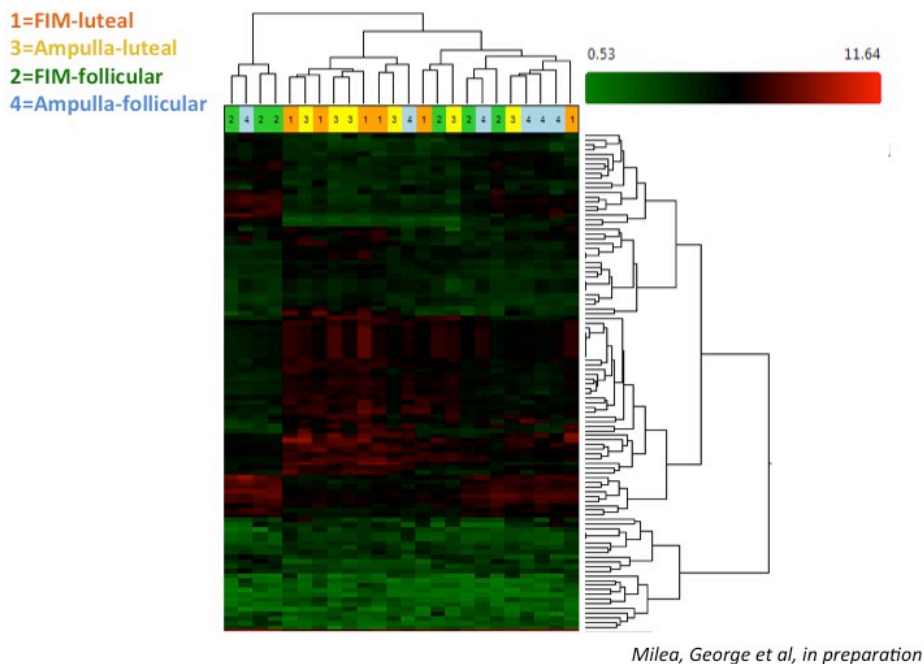


Figure 2. Snap-frozen matched fimbria and ampulla tissues were controlled for age and ovarian cycle status. Cases included 12 luteal phase and 12 follicular phase women at no known risk for ovarian cancer. Laser capture microscopy was used to micro-dissect FTE cells, using 7-10 sections per case. Total RNA was isolated, RNA extracted and cDNA amplified. The expression profiles were generated using Affymetrix Human Genome HTA-2.0 Array.

B). Microdissection of selected paraffin tissue samples

Fimbriae were micro-dissected from 10 luteal and 10 follicular control samples and 8 BRCA follicular and 8 BRCA luteal of the projected total and these samples are ready for submission for the Illumina DASL arrays. We project the submission of all samples to the microarray facility within the next 2 months ~ January 2015.

Task 2. Validate mRNA and protein expression of selected classifier genes.

Progress: In preparation for targets from the genomic profiling of STICs and fimbria gene expression arrays, we are in the process of creating **one** additional cancer TMA containing a large set of 300 HGSC samples with known family history of breast/ovarian cancer and patient clinical history including debulking status, treatment, recurrence and overall survival. This TMA will be useful in assessing the alteration of chosen targets within a larger set of cancers with different family history of cancer, as well as assessing the impact of such targets on clinical outcome.

As the microarray analysis on the FFPE samples has not yet begun, the validations of targets in both normal FTE and STIC are delayed. We anticipate submission of the micro-dissected FFPE RNA within 2 months.

Task 3. Model alterations associated with high-risk tubal epithelium and tubal intraepithelial carcinomas, in vitro.

Progress: The isolation and propagation of primary FTE cells is an ongoing effort in our laboratory. In previous reports we have demonstrated the ability to isolate and propagate FTE with more than 14 primary FTE cell lines established from different patient samples. The primary FTE growth kinetics have already been determined and described in previous reports, with primary FTE propagation in culture exhausted at 5 population doublings and additional expansion (15-30 population doublings) achieved with inactivation of (Rb) and/or p53 hTERT and SV40 large T. To further investigate growth kinetics we have established FTE cell lines

with dominant negative p53 (TP53 R175H or DNTP53) and with short hairpin targeting p53 (sh-p53). In previous reports, we have demonstrated the establishment of 35 FTE cell lines and the careful characterization of immortalized human epithelial cells in terms of molecular alterations, morphological appearance in 2D monolayer, shared epithelial/stromal markers with corresponding fallopian tube tissue, growth ability by population doublings and anchorage independent growth.

In the previous report we discussed the study of C/EBP- β a transcription factor initially determined to be up-regulated in normal FTE of BRCA1- mutation carriers. C/EBP- β is commonly induced by stress and is associated with increased DNA damage. We studied the 1) differential expression of C/EBP- β in serous carcinoma 2) expression of C/EBP- β in precursor lesions (STIC) and 3) modeled the effect of overexpression of C/EBP- β in an *in vitro* fallopian tube cell culture model. C/EBP- β is down-regulated in over 60% of HGSC analyzed, an observation which is also histotype specific as LGSC tumors show high expression levels ($p < 0.01$). 60% of C/EBP- β protein expression exhibited a gradual decrease from the normal FTE to the p53-signatures and the STICs and lost in concomitant HGSC. This indicates an inverse correlation between C/EBP- β and proliferative index. This suggests that C/EBP β regulates cell cycle progression in the FTE possibly through the G2/M checkpoint and is a potential tumor suppressor in HGSC. In short-term cultures of FTE cell lines, over-expression of C/EBP- β did not affect proliferation but may affect long-term growth. High expression of C/EBP- β in FTE cells correlated with decreased vimentin, N-cadherin, ACTA2 and Zeb2 while and in Twist1 and mir21 expression. This data is consistent with the phenotypic effect observed by over-expressing C/EBP- β in cell culture and indicates an involvement of C/EBP- β in mesenchymal-to-epithelial transition during high-grade serous carcinogenesis.

We are in the process of writing a manuscript for peer-review.

Immortalized and transformed cell lines from both FTE and OSE, cells derived from both BRCA1 mutation carriers and non-carriers. In particular, 16 OSE cell lines have been created – 3 from BRCA1 carriers – iOSE390F, iOSE267F, iOSE592F (F=familial ovarian cancer) and non-carriers – iOSE120 and iOSE523. Cell lines have been transfected with hTERT and SV40, and either vectors over-expressing cMYC, hRASV12 or PIK3CA-H1047R were generated

We are now performing *in vitro* assays to compare proliferation, anchorage independent growth and invasion between the BRCA1 and non-BRCA OSE cell lines.

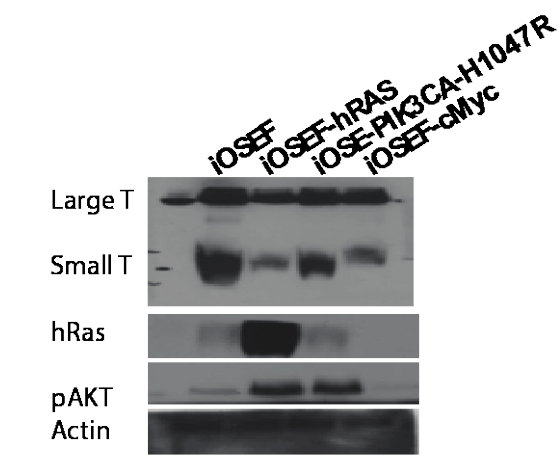


Figure 3. Western blot of iOSE BRCA1 mutation carrier cell line with the over-expression of PIK3CA-H1047R, hRASv12 and cMYC. The blot confirms presence of SV40, over-expression of hRas and the PIK3CA via activation of Akt.

Project 5: Epidemiologic and molecular characterization of putative precursor lesions in the ovary and fallopian tube of unaffected high-risk women.

Specific Aim 1: Using existing samples (collected both in a retrospective and prospective manner) we will; **a)** determine the prevalence, frequency and location of STICs, STILs, p53 signatures, CICs, morphological changes in the OSE and invasive HGSCs in the FTE and ovaries of BSO specimens removed prophylactically in women at high risk for ovarian cancer, overall and stratified by BRCA1/2 mutation status (N ~ 550), **b)** examine correlations between the prevalence of the different types of lesions; and **c)** determine the epidemiological profile including demographics, lifestyle factors and known risk factors for ovarian cancer associated with STICs, STILs, p53 signature, CICs and morphological changes in the OSE.

The Toronto group is responsible for the cutting, staining and shipment of 223 prophylactic cases and 25 high-grade serous ovarian cancer cases. A significant amount of effort has been devoted over the last 6 months toward the completion of this task due to time spent labeling slides, barcode scanning slides, organization into boxes and inventory creation of slides within their respective boxes. To date our group has shipped **164 completed prophylactic cases and 18 completed HGSC cases**. *We have also now, processed 1 HGSC case to be used in Project 1.* We continue to submit tissue blocks for processing to be sent to the storage site at JHU.

KEY RESEARCH ACCOMPLISHMENTS:

- The epithelia of the anatomically high-risk fallopian tube – the fimbria, show few differences in gene expression profiles compared to the lower risk portion – the ampulla. Expression differences predominantly are in response to the hormonal milieu, i.e. the secretory and proliferative phases of the ovarian cycle, and not due to the anatomic site of the epithelium.
- CEBPD over-expression leads to cuboidal phenotypic changes of FTE cells with different pre-malignant permutation types, indicative of a role in mesenchymal-epithelial transition due to its decrease of mesenchymal genes such as Slug and Zeb1.
- We have generated OSE and FTE cell lines from BRCA mutation carriers harboring hRASV12, PIK3CA-H1047R and cMYC

REPORTABLE OUTCOMES:

Publications

1. Sophia HL George and Patricia Shaw. BRCA and Early Events in the Development of Serous Ovarian Cancer. *Frontiers in Oncology*. 2014 Jan 23; 4:5.
2. Milea A, George SH, Matevski D, Jiang H, Madunic M, Berman HK, Gauthier ML, Gallie B, Shaw PA. Retinoblastoma pathway deregulatory mechanisms determine clinical outcome in high-grade serous ovarian carcinoma. *Mod Pathol*. 2013 Dec 13.
3. Sophia HL George, Anca Milea, Ramlogan Sowamber, Rania Chehade, Alicia Tone, Mona Gauthier and Patricia Shaw. Loss of LKB1 Protein Expression is Frequent in Serous Carcinoma. *Under Review, Oncogene* 2014

Abstracts

1. *Origins of ovarian cancer, the biology of premalignancy, and cancer health disparities.* Sophia H.L. George, Judith Hurley, Steven Narod, Talia Donenberg, Mohammed Akbari, Gillian Warfe, Hedda Dyer, Cherly Alexis, Amy Waters, and Anca Milea, Patricia A. Shaw. AACR The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved on December. 6 - 9, 2013 in Atlanta, GA.
2. *Hormone Receptor Status Predicts Clinical Outcome of High Grade Serous Carcinoma: A Gene Expression Study*, Raheem Peeran, Sophia H.L. George, Anca Milea, Ramlogan Sowamber, Danielle Toccalino, and Patricia A. Shaw. The United States & Canadian Academy of Pathology's 103rd Annual Meeting, March 1-7, 2014 in San Diego, CA
3. *BRCA and Early Events in the Development of Serous Ovarian Cancer.* Sophia HL George, Patricia A Shaw. April 23-25, 2014. 5th International Symposium on Hereditary Breast and Ovarian Cancer, Montreal, QC.
4. *The role of BRCA and CEBPD in serous ovarian cancer carcinogenesis.* Sophia HL George, Ramlogan Sowamber, Anca Milea, Noor Salman and Patricia Shaw. September 2014. Masha Rivkin Ovarian Cancer Conference, Seattle, WA
5. *BRCA and early events in the development of serous ovarian cancer.* Sophia HL George, Anca Milea, Ramlogan Sowamber, Danielle Toccalino and Patricia Shaw. Current Oncology 21:e377 (2) April 2014
6. *Evaluation of Bahamian BRCA Founder Mutations in the Caribbean.* Talia Donenberg, Sophia HL George, Mohammad Akbari, Cheryl Alexis, Gillian Wharfe, Sook Yin, Hedda Dyer, Theodore Turnquest, Steven A. Narod, Judith Hurley. Current Oncology 21:e368 (2) April 2014
7. *The role of estrogen receptor signaling in serous ovarian cancer.* Sophia HL George, Anca Milea, Ramlogan Sowamber, Danielle Toccalino and Patricia Shaw. Cancer Research August 14, 2013 73:4765; doi:10.1158/1538-7445.AM2013-4765
8. *Fimbria and Ampulla Tubal Epithelium Have Similar Transcriptome Profiles.* Sophia HL George, Anca Milea, Noor Salman and Patricia Shaw – submitted to USCAP 2015
9. *The Familial Ovarian Tumor Study: A Morphological and Immunological Review.* David Kolin, Sophia George, Anca Milea, Steven Narod, Blaise Clarke, Patricia Shaw – submitted to USCASP 2015
10. *Tumor infiltrating lymphocytes (TILs) as a function of histological subtype and genetic background of ovarian epithelial carcinomas.* Raheem Peerani, Sophia George, Ramlogan Sowamber, Alex Siu, Tiffany Shao, Anca Milea, Steven Narod, Blaise Clarke and Patricia Shaw

Oral Presentations:

Raheem Peerani. *Hormone Receptor Status Predicts Clinical Outcome of High Grade Serous Carcinoma: A Gene Expression Study*, United States & Canadian Academy of Pathology 103rd Annual Meeting, March 1-7, 2014 in San Diego, CA

Sophia HL George. *BRCA and early events in the development of serous ovarian cancer.* Best of ASCO Caribbean. October 18th, 2014, Puerto Rico.

Sophia HL George. *The fallopian tube and early events in ovarian cancer.* June 25, 2014. Ponce School of Medicine, Puerto Rico

Patricia Shaw. *BRCA and Early Events in the Development of High Grade Serous Ovarian Carcinoma.* 5th Annual Ovarian Cancer Symposium – Prevention, Detection and Treatment of Ovarian Cancer, September 22, 2014, Toronto, Canada

The Toronto Group hosted the 5th Annual Ovarian Cancer Symposium in September (22nd-23rd). There were 106 registered participants and 13 individuals submitted abstracts for poster presentation.

CONCLUSIONS:

Through the work over the last 6 months we have begun to understand the biological relevance of genes found to be differentially expressed between BRCA1 and controls in the fallopian tube epithelium. Although still preliminary, we identify C/EBP-delta as a possible regulator of mesenchymal to epithelial transition in the fallopian tube epithelium. As a result of the work presented in the last report and abstract attached we are in the process of writing a manuscript for peer-review publication. Additionally, the work on estrogen and progesterone receptor signaling in the FTE, STIC and HGSC is being prepared for peer-review publication. Our team continues to work on the study of BRCAness in both FTE and now established OSE cell lines and we will present data of this work in the next report.

We have also deduced that the tubal fimbria and ampulla of the tube are very similar at the transcriptional level. We therefore stipulate that the higher-risk area of the tube - the fimbria, is more exposed to DNA damage and effects from the ovulation due its proximity to the ovary. Additionally, we continue to study at the immunohistochemical level, the differences between the histological normal FTE and that in BRCA mutation carriers – using targets obtained from the previous microarray studies and the recent ampulla/fimbria study - as we try to understand the biology determining cancer formation.

REFERENCES:

1. George SH, Greenaway J, Milea A, *et al.* Identification of abrogated pathways in fallopian tube epithelium from BRCA1 mutation carriers. *J Pathol* 2011; 225(1):106-17.
2. Wang J, Sarkar TR, Zhou M, *et al.* CCAAT/enhancer binding protein delta (C/EBPdelta, CEBPD)-mediated nuclear import of FANCD2 by IPO4 augments cellular response to DNA damage. *Proc Natl Acad Sci U S A* 2010; 107(37):16131-6.
3. Krockenberger M, Honig A, Rieger L, *et al.* Transketolase-like 1 expression correlates with subtypes of ovarian cancer and the presence of distant metastases. *Int J Gynecol Cancer* 2007; 17(1):101-6.

APPENDICES:

1. *Origins of ovarian cancer, the biology of premalignancy, and cancer health disparities.* Sophia H.L. George, Judith Hurley, Steven Narod, Talia Donenberg, Mohammed Akbari, Gillian Warfe, Hedda Dyer, Cherly Alexis, Amy Waters, and Anca Milea, Patricia A. Shaw. AACR The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved on December. 6 - 9, 2013 in Atlanta, GA.
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3. *BRCA and Early Events in the Development of Serous Ovarian Cancer.* Sophia HL George, Patricia A Shaw. April 23-25, 2014. 5th International Symposium on Hereditary Breast and Ovarian Cancer, Montreal, QC.
4. Sophia HL George and Patricia Shaw. BRCA and Early Events in the Development of Serous Ovarian Cancer. *Frontiers in Oncology*. 2014 Jan 23; 4:5.
6. Milea A, George SH, Matevski D, Jiang H, Madunic M, Berman HK, Gauthier ML, Gallie B, Shaw PA. Retinoblastoma pathway deregulatory mechanisms determine clinical outcome in high-grade serous ovarian carcinoma. *Mod Pathol*. 2013 Dec 13.

Project 4. Locate and characterize precursor lesions of “ovarian” cancer in a mouse model and explore the role of ovulation and changes in the microenvironment of the ovary and tube in “ovarian” carcinogenesis using human tubal xenografts in nude mice.

Research site: Johns Hopkins University

Project Leader: Tian-Li Wang

Co-investigators: le-Ming Shih (JHU)

Section II. Progress to Date

Task 1. Locate the anatomic site and characterize the precursor lesions of “ovarian” cancer in the TP53^{-/-}/Rb^{-/-} mouse model.

Task 1a. Mouse breeding and genotyping (1-18 months)

Progress: This task has been finished. Please see the following tasks for details.

Task 1b. TP53/Rb KO mice study to locate the anatomic site of precursor lesions (1-15 months).

Progress: We have finished the study related to TP53/Rb KO mice but failed to observe tumor phenotypes in at least 20 female mice for a period of time up to 16 months. Therefore, we have sought to use another mouse model. To this end, in collaboration with Dr. Pat Morin, we have focused on Ovgp-1 Tag mouse model in which the expression of T antigen oncogene is driven the promoter of oviduct glycoprotein 1. Since large T antigen simultaneously inactivates both Rb and p53 pathways, we consider it as a good model to study ovarian serous carcinomas in which mutations and deletions are frequently observed. In fact, we have recently published the characterization of this model and demonstrate that this model is very useful for Project 4 (please see below).

Task 1c. Characterize the mouse precursor lesions using a variety of proposed methods (10-33 months).

Progress: This task has been completed and the data has been published and presented in the last progress report and will not be re-iterated in the current report. *J Pathol*, 2014, Mar 20 doi:10.1002/path 4535 [Epub ahead of print]. PMID:24652535

Task 2. Assess the biological effects of ovarian follicular fluid on human fallopian tube epithelium (FTE) and ovarian surface epithelium in a xenograft model.

Task 2a. Establish the human fallopian tube xenograft model (18-30 months).

Progress: We have made progress in establishing this model and so far we have transplanted at least 15 human normal fallopian tubes into nu/nu mice. Currently, we are characterizing these xenografts and the results will be reported in the next progress report.

Task 2b. Collect human follicular fluid and primary characterization of the fluid (15-25 months).

Progress: This task is in progress. So far, we have obtained two batches of follicular fluids, FT406 and FT2826. Each batch came from 6 women whose follicular fluids were pooled. We have used these two batches of follicular fluids for initial characterization (see the following Task 2C).

Task 2c. Assess the biological effects of ovarian follicular fluids on FTE and OSE in a xenograft model (30-48 months).

Progress: We have collected human follicular fluids and human tubal fluids and tested their DNA damaging effects on FT cultures. As described in the last progress report, we have performed the comet assay and gH2A staining and Western blot analyses to quantitate the damages in double strand DNA. We will continue this task and determine if this task is informative to reveal underlying biology of ovarian cancer development.

Task 3. Determine whether oral contraceptives (OCPs) and NSAIDs reduce the morphologic and molecular changes that are associated with early “ovarian” carcinogenesis.

Task 3a. To assess whether OCPs decrease the frequency of precursor lesions and/or delay tumor development (24-48 months)

Progress: We have finished this task and found that there was no difference in the experimental and control groups after exposure of OCPs.

Task 3b. To determine the effects of aspirin on reducing oxidative stress-induced molecular changes on human fallopian tube and/or on OSE (24-60 months).

Progress: As discussed with the advisory board members and other consortium investigators, we have changed our priority by focus on determining if the statin drugs have a preventive effect on developing ovarian cancer and its precursor lesions using the OVGP1 mouse model. This is because statin drug has come to the research spotlight in cancer therapy and our preliminary results show a significant reduction of tumor progression in mouse tumor xenograft model. We are currently setting up experiments to test if statin drug can have an anti-tumor effect on the OVGP1 mouse model and we expect to report preliminary results in the next report. In the last year, we have made substantial progress in this project and have wrapped up a draft of manuscript as attached. Therefore, please refer to the appendix for the detailed findings from this part of research.

Task 3c. Data analysis and preparation for publication (24-60 months).

Progress: We are going to submit the manuscript of the OVGP1-statin mouse model.

Section III. Problem Areas for Project 4

There are no major problem areas noted for Project 4.

Section IV. Future Work in Project 4

Our main objective in the coming year is to focus on the most exciting finding in Project 4- i.e., the chemoprevention of STIC by statin drugs. We will submit the manuscript in the coming one or two months and as expected, will revise our manuscript according to the reviewers' comments.

Project 5: Determine the molecular and epidemiologic profile of putative precursor lesions in the fallopian tubes and ovaries from women at high-risk for ovarian cancer. In addition, Project 5 will determine if these biomarkers and associated precursor lesions are modifiable by oral contraceptives (OCPs) or anti-inflammatory agents, as OCPs in particular are known to prevent ovarian cancer and impact survival.

Research Site: Johns Hopkins University

Principal Investigator: Kala Visvanathan, MD, MHS

Collaborating Sites: University of Toronto (Steven Narod, MD and Patricia Shaw, MD: Co-investigators), Memorial Sloan Kettering Cancer Center (Douglas Levine, MD and Robert Soslow, MD: Co-investigator), Yale University (Vinita Parkash, MD and Ellen Matloff, MSc, MS, CGC: Co-Investigators)

Section II. Progress to Date

We continue to work intensively on completing the revised Aim 1. We have identified 106 eligible cases from Johns Hopkins, MSKCC, and Yale. (Toronto is not involved in this part because the pathology department would not allow them to flip the blocks and sample from both ends.) All the blocks have been requested at the various sites. Slides have been made from the top and bottom of each fallopian tube and ovarian tissue block for all cases at Hopkins, greater than half at MSKCC and greater than half at Yale. Five slides are cut from the top and bottom of each block. At the same time immunohistochemical staining for multiple markers (p53, Ki67 and Laminin C) as well as H& E stains for each set of slides is ongoing. At Johns Hopkins slides have been cut from all tissue blocks of the 37 cases and IHC staining is nearing completion. Redacted pathology reports are uploaded for these cases in the Path Core/Biorepository database as well. Sloan Kettering has cut and sent slides for 16 of their 33 pilot cases and are in process of finalizing the other 17 sets for sending. They have uploaded redacted pathology reports to the Path Core/Biorepository database as well. Yale encountered a few staffing/work load issues during the past few months but these are resolved. As a result they recently sent slides for approximately 20+ pilot cases and expect to have the remainder sent to the Path Core/Biorepository by the end of November. Our goal is to complete all staining by end of January. This is a massive project. One case has approximately 120+ slides to be stained. At the same time we have started the review process. This involved creating a review sheet, organizing the slides and having two GYN pathologists review the sets.

We also are making sure we have the redacted pathology reports and entered demographic and minimal clinical data on these cases. We hope to have the data to analyze by Feb/March.

This will be the first study to so intensively evaluate the fallopian tube for STILS, STICS and P53 signatures as well as the ovaries. It will also be the first study to look at all these markers. Given the huge amount time required for staining etc. we will also evaluate the first 37 cases to see if we can minimize the number of markers tested in subsequent cases. As indicated previously, the majority of the cases used in the pilot study will also be used in Aims 2 and 3.

We are now focused on obtaining epidemiological data on ongoing cases at the various institutions so that once we have completed the pilot we can sample these cases and conduct the more detailed epidemiological analyses outlined in Aim 2 and Aim 3. We have cleaned the initial set of data from MSKCC and Toronto and Hopkins. We are now re-contacting them to get additional data to meet or exceed our initial sample size given the low prevalence of these lesions. We expect this to be complete in 6 months and will be ready to sample.

We expect to publish the pilot in the next 6-8 months and also begin on marker studies (Aims 2 and 3) with publications quickly following.

Task 1. Obtain approval for the addition of questions for prospective collection from all site IRBs (Aim 1) and also for the transfer of existing epidemiological and clinical data to JHH de-identified for the retrospective study (Aim 1 and 2). Obtain approval from USAMRAA for human subject's research for entire protocol (Months 1-6).

Progress: Approvals have been obtained for all the retrospective and are in process for the prospective collection. We plan to move forward now that the pilot study is underway.

Task 2. Identification of study population for retrospective study based on eligibility criteria from all sites (Months 1-6).

Progress: There has been substantial progress on this aim. Epidemiological data and pathology reports on over 668 cases have been identified. Data entry is still ongoing for approximately 100 at this stage. We continue to work with Yale to obtain some additional funding. The path reports of these cases have also been uploaded. This has required close collaboration between the epidemiologist/clinician and pathologist at each site. Data cleaning is under way with the master epidemiological data at Johns Hopkins.

Task 3. Comprehensive pathology review of samples. A comprehensive master dataset that includes epidemiological and pathological data from approximately 550 women to be used for cross-sectional and case control study designs will be created (Months 6-18).

Progress: This is on hold for the master dataset until after the pilot is finished.

Task 4. Analyses for Aims 1a and b will be completed to determine the prevalence, location and frequency of the specific lesions in the FTEs (STICs, STILs, and p53 signatures) and OSE, as well as CICs in the ovaries, overall and by BRCA mutation status. Correlations between each of the different types of lesions will be determined. Manuscript preparation Aims 1a and b (Months 6-18).

Progress: This Aim has been modified as described above. We are now confident that the pilot study will be completed in the next 8 months and published and we will be able to move quickly on the larger study.

Task 5. Analyses for Aim 1c the association between exposures (both risk and protective factors for ovarian cancer) and each type of prevalent lesion will be examined through cross-sectional studies. Manuscript preparation for Aim 1c (Months 12-24).

Progress: Pending results of pilot study. Infrastructure is being put in place already including identification of cases and input of pathology reports and epidemiological data.

Task 6. As described above, merging of epidemiological and pathological data for aims 1 and 2 will be performed and a comprehensive master dataset to be used from which matched case-control studies will be identified for Aim 2 (Months 6-18).

Progress: Ongoing

Task 7. Case-control sets for molecular analyses of the FTE and ovary samples from Aim 1 will be used to assess a panel of markers. Cases will be defined by the lesion/region of interest (i.e. STICs, STILs, p53 signatures, CICs, and/or morphological changes in OSE). Controls will vary depending on the analysis: marker expression within a specific lesion will be compared to (1) adjacent normal tissue from the same case and (2) normal tissue from women with no identifiable lesion. For the latter comparison, 2 controls will be matched to each case from the same research site, within +/- 2 years of age at the time of surgery. To conserve this valuable tissue, and maximize efficiency, the same controls will be reused for subsequent case-control analyses where possible (Months 18-24).

Progress: Pending

Task 8. Molecular analyses to determine molecular profile of each lesion type will be performed on case control sets. The resulting laboratory results will be merged with existing epidemiological data and analyses will be performed (Months 24-48).

Progress: Our first molecular analysis will be with the pilot study and then we will move on to the larger study.

Task 9. Statistical analyses of case-control sets will be completed for each lesion type and panel of markers. Multiple manuscripts will be generated from Aim 2 based on the different lesions and also markers. These will be prepared and submitted (Months 24-48).

Progress: Pending

Task 10. High-risk women considering BSO in the next 2 years, and meeting the same eligibility criteria used in Aims 1 and 2, will be prospectively enrolled at each site. Information on NSAIDs, OCP and Vitamin E use as well as other ovarian cancer risk/protective factors will be collected through questionnaires completed within 2 years of surgery (N ~ 300-400) (Months 6-48).

Progress: We plan to re-contact cohorts in the next 2-3 weeks to obtain additional cases. We currently have both retrospective and prospective data.

Task 11. Ongoing comprehensive pathological review and merging of epidemiological and pathological data will occur (Months 6-50).

Progress: This is about to begin with the pilot study samples and then will be followed by the larger data set.

Task 12. Complete merging and data management of Master data set (Months 48–50).

Progress: Ongoing

Task 13. Analysis of prospective data with respect to NSAIDs and OCP use will be completed: a) Associations between OCP/NSAID/Vitamin E use and prevalence of lesions will be evaluated, overall and stratified by BRCA mutation status as in Aim 1, and b) associations between use of these substances and molecular markers identified in Aim 2. Manuscripts for Aim 3 will be prepared and submitted. This aim will be informed from data generated in Aims 1 and 2 (Months 48-60).

Progress: We currently have both prospective and retrospective data so we are evaluating the use of these medications in this data.

Section III. Problem Areas of Project 5

The major limitations has been the amount of work involved in such a comprehensive and detailed tissue study. This includes the identification of cases, inputting of data, extensive sampling of tissue blocks, staining for multiple markers and pathology review. We have had to develop new and innovative approaches along the way.

Section IV. Future Work in Project 5

In the next 8 months we hope to complete the pilot study. This will be the first study to characterize in detail the location and the prevalence of STICs in the fallopian tube and ovary. It will also help us determine a less intensive but feasible sampling strategy for sampling the tissue from the 500-700 women that are part of project 5. In addition it will inform clinical practice, as there is currently no standardized protocol on how to best sample for STICs

We will have all the clinical data cleaned and pathology reviews inputted also in real time so that we can analyze and write the manuscript as soon as the data is available.

In the next 6 months we will also continue to expand the master epidemiological data. We will continue to input epidemiological data on potential cases and controls as well as pathological records both from retrospective and prospective collections.

Administration (Admin) Core, Biostatistics/Bioinformatics/Epidemiology (BBE) core, and Pathology (Path) Core

As described in the previous progress reports, the integration of the three cores has been integrated to each other; therefore, we combine their progress in this section to avoid reiteration. Because the tasks related to the three cores are relatively generic and are applicable for the entire research period, so we will rather report the specific progress related to the cores.

Section II. Progress to Date:

In the last 6 months, the Administration Core led by Drs. Kurman and Shih continues providing all the administrative support to all 5 research projects. Communications among project leaders have become a routine through several venues as described in previous reports. The Administration Core has help organized the Ovarian Cancer Symposium has been held in Toronto this September and was a highly successful one with more than 200 anticipated attendees. All the advisory board members attended this meeting and gave insightful advice to all the project leaders. As in the previous symposia, this Toronto Symposium highlighted the major progresses made by the DoD consortium investigators and timely update of new advances in the ovarian cancer research field.

The BBE core provides excellent support to the current study design and assists statistical analysis for all the data generated in the past 6 months. The BBE core is currently working with investigators to prepare for manuscripts to be submitted to journal publications in the future.

The Path Core led by Drs. Visvanathan, Soslow, Kurman and Shih continues to serve as our central collection resource for the various projects. Highlights of Path Core progress this past period include:

1. Demographic and clinical data continue to be collected and entered in the Path Core for the various projects as new cases are submitted.
2. Slides for Project 1 and Project 5 continue to be cut and sent for inclusion in the database (total of 28,539 slides entered in the database as of 11/5/14).
3. Pathology Reports for Project 1 and Project 5 continue to be redacted and uploaded to the database (total of 429 path records have been uploaded as of 11/5/14).

	PROJECT 1				PROJECT 5				
SITES	# OF CASES IN PATH CORE	PATH REPORT UPLOADS	STAININGS FOR P53 AND Ki67	STAINING FOR LAMC1	# OF CASES IN PATH CORE	PATH REPORT UPLOADS	STAININGS FOR P53 Ki67 and H & E	STAINING FOR LAMC1	TOTAL SLIDES (STAINED & UNSTAINED) for P5
JHU	23	23	23	23	37	37	3,216	1,072	5,360
MSKCC	15	15	15	15	40	134	1,200	0	2,009
YALE	10	0	10	10	47	0	in progress	0	6,080
TORONTO	25	25	25	25	184	195	0	0	17,078

4. Database modifications were made to the slide review process section and several data base queries have been developed for reporting and management purposes.

5. Continuing Approval was granted by the JH local IRB for the Path Core/Ovarian Biorepository on October 24, 2014. This will be reported in our next DoD HRPO Report.

6. The slide re-review process for Project 1 cases and as well as Project 5 pilot study cases is underway and expected to be completed in the upcoming three months.
7. Path Core staff continue to provide ongoing support with the sites via email and phone calls regarding bar code issues, data and/or slides submission issues, or general database access questions.

Section III. Problem Areas for the Cores

We are expecting that we will not have sufficient fund for the next Ovarian Cancer Consortium meeting. The Admin Core will suggest that individual investigators may need to cover part of their cost to attend the meeting. Otherwise, we do not expect major problems for the three cores.

Section IV. Future Works in Cores

The future works for the three cores correspond to what have been proposed in this consortium study. Briefly, the Admin Core will continue overseeing and manage performance of the Path and BBE Cores and the Research Sites. Specifically, the Admin Core will promote the integration and coordination of research activities, ensure new IRB protocols and additional biosafety regulations are in place, coordinate intellectual and material property, organize regular meetings of the investigators including the Annual Ovarian Cancer Symposium, monitor project performance, prepare annual progress reports, organize and make travel arrangements for investigators and ensure dissemination of data and publication of major findings as well as maintaining the ovarian cancer web site. The Admin Core will plan the next Ovarian Cancer Symposium and this task will be the priority for this Core. The Path Core will be responsible for tissue repository and the BBE core will ensure that data are consistently collected and entered into the Consortium database. As described in the task, the Path Core will collect tissue and analyze it histologically according to standardized practices and the algorithm that we developed for this program, perform tissue-based assays, and annotate this material in a centralized fashion while assuring quality. The Path Core will also oversee functions related to storage and transportation of tissue samples in order to supply researchers with pathologically characterized material. Both Path and Admin Cores will work together to coordinate the shipment of slides among Research Sites and the Pathology Core. The BBE Core will provide biostatistics, bioinformatics, and epidemiology consultation and support to all members of the Consortium. Core members will assist in the design, collection, storage, visualization, analysis, quantitative modeling, and interpretation of the data arising in the course of Consortium activities. Centralizing biostatistical and bioinformatics support within the Core will ensure that the necessary expertise will be available for all projects, facilitating the coordination and integration of data throughout the Consortium in a consistent fashion. Dr. Cope in BBE core will analyze the profiling data collected from Project 2.

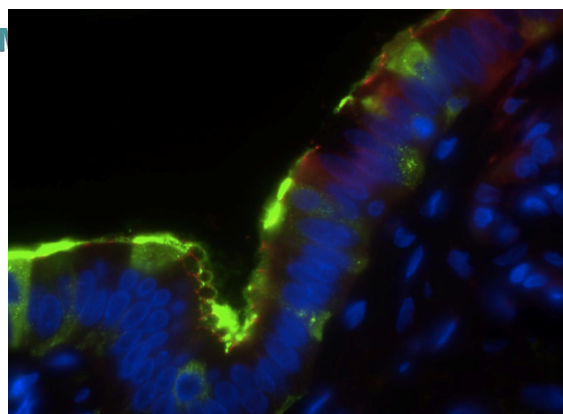
Section V- Administrative Comments (Optional)

Section VI- Meetings

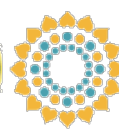
We will schedule the next DoD Consortium PI meeting in conjunction with the Ovarian Cancer Symposium in 2015. EAB and RCA meetings will also be scheduled at that time.

Prevention, Detection and Treatment of OVARIAN CANCER

September 22-23, 2014



Princess Margaret Cancer Centre
610 University Ave
6th floor Robert Bell Auditorium



Ovarian Cancer Canada
Cancer de l'ovaire Canada

Guest Faculty

Carrie Rinker-Schaeffer, PhD

Professor, Department of Surgery
University of Chicago

Cynthia Zahnow, PhD

Associate Professor of Oncology
Johns Hopkins University

Douglas A. Levine, MD, FCOG, FACS

Associate Attending Surgeon Head Gynecology
Research Laboratory
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Ie-Ming Shih, MD, PhD

Richard W. TeLinde Distinguished Professor
Dept of Gynecology and Obstetrics, Johns
Hopkins University

Kala Visvanathan MBBS, FRACP, MHS

Associate Professor in Medical Oncology and
Epidemiology
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Mark E. Sherman, MD

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Tian-Li Wang, PhD

Associate Professor, Dept of Pathology
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William Foulkes, MD, PhD, FRCPC

Professor, Depts of Medicine
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Amit Oza, MD, FRCPC

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Assistant Professor
Laboratory Medicine and Pathobiology

Pamela Ohashi, PhD, FRCS

Professor, Medical Biophysics

Robert Rottapel, MD

Professor, Medical Biophysics

Patricia A Shaw, MD, FRCPC

Professor, Laboratory Medicine and Pathobiology

Monday, September 22

- 9:30 Opening remarks
9:35 Introduction to DOD Consortium

Session 1: Prevention and Early Events in Ovarian Cancer

- 9:40 Robert Kurman, MD
Ovarian Cancer. The Long and Winding Road to Prevention
10:15 Patricia Shaw, MD, FRCP
BRCA and Early Events in the Development of Serous Ovarian Cancer

10:45 Break

- 11:20 Mark E. Sherman, MD
High-Grade Serous Carcinoma: Unanswered Questions about Pathogenesis
11:50 Blaise Clarke, MD
Lynch Syndrome and Ovarian Cancer

12:15 Lunch and Poster Session -7th Floor Atrium

Session 2: Genomics and Models of Ovarian Cancer

- 1:45 Ie-Ming Shih, MD, PhD
The roles of ARID1A in the pathogenesis of Type I ovarian cancer
2:15 Benjamin Neel MD, PhD
Mathematical Model of Ovarian Cancer Therapy
2:45 Carrie Rinker-Schaeffer, PhD
Dark Passage: Discerning Mechanisms Regulating the Development of Peritoneal Metastases

- 3:15 Break
3:30 Robert Rottapel, MD
Untangling the Functional Genetic Architecture of Ovarian Cancer
4:00 Douglas Levine, MD, FACOG, FACS
Genomic profiling and clinical outcome of STIC associated ovarian carcinoma
4:30 William Foulkes, MD, PhD
Inherited factors in non-epithelial ovarian cancer
5:00 End of day

Tuesday, September 23

Session 3 - Prognosis and Treatment of Ovarian Cancer

- 8:30 Cynthia Zahnow, PhD
The use of epigenetic therapy to augment immune signaling and sensitize ovarian cancer to immune checkpoint inhibitors.
9:00 Amit Oza, MD, FRCP
Molecular stratification in treatment of ovarian cancer
9:30 Pamela Ohashi, PhD
Inhibitory mechanisms in the ovarian cancer tumor microenvironment

10:00 Break

10:15 Tian-Li Wang, PhD
Developing small compound inhibitors for chemoresistant ovarian cancer
10:45 Robert J. Kurman, MD
Serous Borderline Tumors at 50. What Have We Learned?

11:15 End of symposium



Ovarian Cancer Canada
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Abstracts

#1. A new approach to inhibit the immunosuppressive properties of galectin-7 in ovarian cancer

Maria Claudia Vladoiu, Marilynne Labrie, Myriam Létourneau, Donald Gagné, Andrée-Anne Grosset, Étienne Billard, Nicolas Doucet, David Chatenet, Yves St-Pierre.

Ovarian cancer (OC) is one of the biggest killers and the second most commonly diagnosed gynecological cancers in North America. Such devastating statistics are in part due to the late diagnosis of the pathology since symptoms generally occur in advanced-stage metastatic tumors. Increasing evidence suggests however that a big component involved in OC progression is played by the immune system. Specifically, the presence and activity of tumor infiltrating lymphocytes (TILs) has been associated with a better overall survival in OC. As such, active and well-functioning TILs, namely CD3⁺ and CD8⁺ seem to play important roles in the progression and potentially in the elimination of ovarian cancerous cells. Conversely, galectins are a class of proteins capable of immuno-modulation and have been found in the extracellular environment of certain tumors. In recent years, their ability to bind and stabilize signal transduction on cell surface receptors through their dimerization has become of great interest. This feature seems to play an essential role in cancer as it can stabilize the expression of membrane receptors and thereby promote tumor growth leading to the formation of a local immunosuppressive microenvironment through the induction of T-lymphocytes apoptosis. In fact, immunohistochemical studies have shown that a high expression profile of galectin-7 is associated with a higher mortality rate and a poor overall survival of OC patients. In addition, our lab has demonstrated the induction of apoptosis in T-lymphocytes and monocytes due to the presence of extracellular galectin-7. Like most galectins, galectin-7 can enable the formation of a homodimer through the noncovalent interaction of its individual monomers. Using galectin-7 as a prototype, the overall objective of our project is to develop inhibitors that interfere with the formation of its homodimer and test their potential to inhibit the immunosuppressive activity of galectin-7. To achieve this, we generated a series of peptides that could interfere with the dimerization site interphase of the protein and studied their functional properties.

The generated peptide (pGal7) has demonstrated efficacy in disrupting the homodimeric structure of galectin-7's recombinant protein. This effect was seen not only in the potassium phosphate buffer (pH 7.1) and RPMI media, but also in the lactose solution (0.1mM) suggesting that the process by which pGal-7

disrupts the dimeric formation of galectin-7 is CRD independent. Furthermore, the unaffected dimer formation of galectin-1 in the presence of pGal-7 implies that the peptide interaction with galectin-7 is specific. In addition, an increase in the binding of galectin-7 to the surface of T lymphocytes, particularly the Jurkat cell line was observed in the presence of pGal7. This observation proposes that the increase in binding may be due to the increase presence and availability of galectin-7 monomers generated by the presence of pGal7. Finally, we also observed a decrease in the apoptosis induced by galectin-7 in the presence of pGal7 not only in our cell-line model but also in T lymphocytes isolated from human peripheral blood. This suggests that the formation of the galectin-7 dimers is the key to intracellular signaling of apoptosis in these cell types. Our model provides a new approach allowing to deepen our understanding of galectin's homodimeric structure, but also to consider the development of new peptide inhibitors that could aid in OC therapy.

#2. Galectin-7 increases the invasive behavior of ovarian cancer cells and promotes tumor immune escape

Marilynne Labrie, Maria Claudia Vladoiu, Andrée-anne Grosset, Louis Gaboury and Yves St-Pierre

Ovarian cancer (OC) is the 5th leading cause of cancer-related deaths in the Western world, the second most common gynecological cancer and the leading cause of death from gynecological malignancies. Here, we have investigated the role of galectin-7 (gal-7) in OC. Gal-7 is an epithelial cell marker which has been shown to play an important role in the progression of many types of cancer, including breast and colon cancers. Galectins play an important role in several physiological processes, including cell migration, apoptosis and regulation of the immune response. They are also involved in a number of pathological conditions, including cancer. In fact, galectins have been shown to create an immunosuppressive tumor microenvironment by killing helper and effector T cells. They can also bind to the surface of tumor cells to form cell surface lattice that stabilizes the expression of key growth factor receptors, thereby increasing cell growth, motility and production of matrix metalloproteinases. Using tissue microarrays, we found that gal-7 expression increases in malignant tumors compared to normal tissues and benign tumors. Detection of gal-7 was also more frequent in tissue samples with metastasis as compared to non-metastatic primary tumor samples. Western blot analysis showed that OVCAR-3 OC cells secrete gal-7, while no expression was detected in A2780 and SK-OV-3 cell lines. Treatment of OC cells with recombinant gal-7 increased their invasive behavior through matrigel and their ability to

express MMP-9, an extracellular proteases which confers an invasive phenotype to cancer cells. Treatment of freshly isolated human PBMCs with gal-7 also showed an increased apoptosis of monocytes, T CD4+ and T CD8+ cells. Overall, these data provide impetus for further studies to determine the potential of gal-7 as a therapeutic target for the treatment of OC.

#3. RUNX3 AND WNT—DO THEY RENDER OVARIAN CANCER CELLS CARBOPLATIN-RESISTANT?

Samir H Barghout¹, Nubia Zepeda², Abul K Azad², Zhihua Xu^{1,2}, Christine Yang², Helen Steed^{1,2}, Lynne Postovit², YangXin Fu^{1,2}

Department of Obstetrics and Gynecology¹, Department of Oncology², Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada.

Objective: Ovarian cancer is the leading cause of mortality due to gynecologic malignancies. Approximately 90 % of ovarian malignancies fall into the epithelial ovarian cancer (EOC) type. However, chemotherapy regimens currently employed are ineffective against advanced EOC because of chemoresistance. Identifying the molecular mechanisms underlying chemoresistance will therefore help to develop more effective therapeutic strategies.

Methods: Microarray & Ingenuity Pathway Analysis (IPA), qRT-PCR, Western blot, cytotoxicity assays and luciferase reporter assay.

Results: The gene expression profile analysis showed that RUNX3 expression is elevated and Wnt signaling is more active in cisplatin-resistant A2780cp cells compared to the cisplatin-sensitive counterpart A2780s cells. Overexpression of RUNX3 renders A2780s cells more resistant and inactivation of RUNX3 by dominant-negative RUNX3 moderately sensitizes A2780cp cells to carboplatin. Down-regulation of the endogenous Wnt inhibitory proteins (DKK1, SFRP1 and FRZB) and up-regulation of Wnt ligands (WNT3, WNT11 and WNT3A) and Wnt target genes (*JUN*, *CCND1*, and *AXIN2*) in A2780cp cells compared to A2780s cells suggest that Wnt signaling is more active in A2780cp cells. Using the luciferase reporter assay, we confirmed that β -catenin transcriptional activity is higher in A2780cp cells than in A2780s cells. Additionally, combined treatment of carboplatin and CCT036477 (a β -catenin inhibitor) was more effective in killing A2780cp cells than either agent alone.

Conclusion: Our data suggest that RUNX3 and Wnt/ β -catenin signaling contribute to carboplatin resistance of A2780cp cells. Therefore, RUNX3 and the Wnt/ β -catenin signaling pathway may represent

attractive targets to overcome resistance of EOC to carboplatin.

#4. Patient-derived Xenografts as a Tool for the Identification of Tumour Secreted Proteins for High Grade Serous Ovarian Carcinoma

Ankit Sinha, Ali Hussain, Kwan Ho Tang, Vladimir Ignatchenko, Ben Neel, Laurie Ailles and Thomas Kislinger

The current FDA approved biomarker for detecting recurrence of ovarian cancer is the cell-surface glycoprotein CA125. However, a recent randomized trial failed to conclude an improvement in patient survival on the basis of treatment with rising CA125 levels against symptomatic recurrence. Hence, we hypothesize that identification of additional markers (potentially used as a panel) will improve analytical sensitivity for surveillance of HSOC recurrence. To identify such HGSOE tumour secreted proteins in serum, we are profiling N-glycosylated proteins present in tumour and serum of patient-derived xenografts. Analysis of matched xenograft tumor tissue and serum established from recurrent high-grade serous cases has resulted in the detection of 1500 unique glycosylated peptides in xenograft tumor tissue, and around 300-500 unique glycosylated peptides (along with CA125) in xenograft serum. We developed bio-informatic approaches to determine the biological source of the identified peptides as originating from human tumour or mouse stroma/tissue. Alternatively, identified peptides could be sequence conserved between human and mouse. Comparison to un-engrafted serum will provide an additional control for the prioritization of potential candidates. By merging N-glyco-proteomics and the qualitative ability of mass spectrometry to detect peptides of human origin within a complex mouse background, we expect to target a sub-class of secreted proteins with emphasis on potential tumour markers.

#5. The Role of Stromal Cells in Supporting Tumour Initiating Cells in Serous Ovarian Carcinoma

Ali Hussain¹, Benjamin Neel^{1,2}, Laurie Ailles^{1,2}

¹Department of Medical Biophysics, University of Toronto ²Ontario Cancer Institute and Campbell Family Institute for Cancer Research, Toronto.

High grade serous ovarian carcinoma has been shown to be a highly heterogeneous disease. *In vivo* studies demonstrate that the tumor initiating frequency varies substantially from case to case. Nonetheless, the tumor initiating subset remains a rare population within the epithelial compartment and can be enriched using the cell surface marker CD133. The tumor microenvironment may play a role in

supporting and maintaining tumor initiating cells through direct and/or indirect cross talk. The contribution of the mesenchymal component of the microenvironment may prove to be essential in providing such support as has been previously shown in studies on breast and prostate cancers.

We have derived and established cancer-associated fibroblast (CAF) lines from bulk primary serous ovarian tumors. A profile of surface markers expressed on the surface of these CAF lines was then generated by running a flow cytometry-based high throughput screen for 370 known cell surface proteins. Our top candidate fibroblast marker was then validated through analytical FACS on 20 primary patient samples, and immunohistochemical staining of primary tumor tissue. In all of those patient samples, the staining profile was comprehensive and specific to the stromal population. Moreover, functional validation of the influence of stromal fibroblasts on the growth of tumor cells is currently being investigated through co-injection and co-culture assays. Preliminary data show that the presence of fibroblasts better supports the growth of epithelial colonies in culture than when compared to other conditions.

Interplay between the niche and a specific subset of epithelial cells may promote those cells to become more tumorigenic. Such an interaction may be dependent on direct physical contact and/or indirect cross talk. Ultimately, we aim to understand the mechanisms that govern these interactions.

#6. PI3K Inhibitors Sensitize ARID1A-Mutated Cancers to DNA Damaging Agents

Youngran Park¹, Yohan Suryo Rahmanto³, Tae Mogami², le-Ming Shih³ and Tian-Li Wang³

¹Pathobiology Graduate Program, Johns Hopkins University School of Medicine, ²Yokohama City University Hospital,

³Johns Hopkins Medical Institution Department of Pathology

INTRODUCTION

ARID1A (AT Rich Interactive Domain 1A), a tumor suppressor gene encoding a SWI/SNF chromatin remodeling factor, is frequently mutated in endometriosis-associated ovarian cancer including ovarian clear cell carcinoma (CCC) and ovarian endometrioid carcinoma. Loss of ARID1A expression either due to inactivating mutations of ARID1A or epigenetic silencing has been reported to be correlated with chemoresistance in patients with ovarian CCC. Therefore, developing novel therapeutics targeting ARID1A-mutated cancers may overcome chemoresistance associated with ARID1A

mutation, at least in this type of cancer. This study is undertaken to test efficacy of PI3K inhibitors in combination with DNA damaging agent in ARID1A-mutated versus ARID1A-wildtype cancers.

MATERIALS AND METHODS

Isogenic ARID1A-KO and ARID1A-WT cell lines including HCT116 and MCF10A were employed in this study. Cells were challenged with DNA damaging agents including γ -irradiation and carboplatin. Additional cell lines employed including human endometrial epithelial (hEM) cell line, ovarian surface epithelial cell line (OSE4) and carboplatin-resistant (CR) ovarian cancer cell line. Down-regulation of ARID1A was achieved by siRNA and cell cycle progression was determined by propidium iodide staining and flow cytometry.

RESULTS

g-irradiation resulted in higher expression levels of phosphorylation AKT (pAKT) in ARID1A-KO HCT116 cells than HCT116 cells with WT ARID1A. Similarly, knockdown of ARID1A in hEM and OSE4 cells led to increased levels of pAKT. Furthermore, as compared to ARID1A-WT cells, ARID1A-KO cells were more sensitive to PI3K inhibitor in combination with g-irradiation. In carboplatin-resistant ovarian cell lines, increased level of pAKT was observed in ARID1A-KO cancer cells than in ARID1A-WT cancer cells, indicating hyperactivation of PI3K/AKT pathway in chronic DNA damaging condition. The PI3K inhibitor sensitized ARID1A-KO carboplatin-resistant cell line more potently than the ARID1A-KO, carboplatin-sensitive cells or ARID1A-WT cells. Cell cycle analysis demonstrated that ARID1A-KO cells escape G2/M cell cycle arrest after g-irradiation and the activation of checkpoint proteins including pATR and pChk2 were suppressed.

CONCLUSION

- (1) Our data suggest ARID1A may participate in DNA damaging repair pathway.
- (2) PI3K inhibitors sensitize ARID1A-mutated cells to DNA damaging agents including g-irradiation and carboplatin. Therefore, future works are needed to test this combination therapeutics in patients whose tumors harbor ARID1A mutation.

#7. Frequent somatic mutations of the telomerase reverse transcriptase promoter in ovarian clear cell carcinoma but not in other major types of gynaecological malignancy

Ren-Chin Wu,^{1,6} Ayse Ayhan,³ Daichi Maeda,⁴ Kyu-Rae Kim,⁵ Blaise A Clarke,⁷ Patricia Shaw,⁷ Michael Herman Chui,⁷ Barry Rosen,⁷ le-Ming Shih^{1,2,*} and Tian-Li Wang^{1,2}

¹Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD, USA ² Departments of Gynecology and Obstetrics and Oncology, Johns Hopkins Medical Institutions, Baltimore, MD, USA ³ Department of Pathology, Seirei Mikatahara General Hospital, Hamamatsu, Japan ⁴ Department of Pathology, Graduate School of Medicine, University of Tokyo, Japan ⁵ Department of Pathology, Asan Medical Centre, Seoul, Korea ⁶ Department of Pathology, Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taoyuan, Taiwan ⁷ Department of Laboratory Medicine and Pathobiology, University of Toronto, and Department of Pathology, Toronto General Hospital, University Health Network, Toronto, Canada

Up-regulated expression of telomerase reverse transcriptase (TERT) and subsequent maintenance of telomere length are essential in tumor development. Recent studies have implicated somatic gain-of-function mutations at the *TERT* promoter as one of the mechanisms that promote transcriptional activation of TERT; however, it remains unclear whether this genetic abnormality is prevalent in gynecological neoplasms. We performed mutational analysis in a total of 525 gynecological cancers, and correlated *TERT* promoter mutations with clinicopathological features. With the exception of ovarian clear cell carcinomas, in which mutations were found in 37 (15.9%) of 233 cases, the majority of gynecological malignancies were wild-type. *TERT* promoter mutation does not appear to be an early event during oncogenesis, as it was not detected in the contiguous endometriosis associated with ovarian clear cell carcinoma. Ovarian clear cell carcinoma cell lines with *TERT* promoter mutations exhibited higher TERT mRNA expression than those with wild-type sequences ($p = 0.0238$). *TERT* promoter mutation tended to be mutually exclusive with loss of ARID1A protein expression ($p = 4.4 \times 10^{-9}$) and *PIK3CA* mutation ($p = 0.0019$) in ovarian clear cell carcinomas. No associations with disease-specific survival were observed for ovarian clear cell carcinoma. The above results, in conjunction with our previous report showing longer telomeres in ovarian clear cell carcinomas relative to other types of ovarian cancer, suggests that aberrations in telomere biology may play an important role in the pathogenesis of ovarian clear cell carcinoma.

#8. Inhibition of Spleen Tyrosine Kinase Potentiates Paclitaxel-Induced Cytotoxicity in Ovarian Cancer Cells by Stabilizing Microtubules

Yu Yu¹, Stephanie Gaillard¹, Tai-Chung Huang², Sneha M Pinto², Akhilesh Pandey^{1,2}, Jude M. Phillip³, Denis Wirtz^{1,3}, Tian-Li Wang¹ and Ie-Ming Shih^{1,4}

¹Department of Pathology and Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Medical Institutions, Baltimore, Maryland; ²Department of Biological Chemistry and Oncology, Johns Hopkins Medical Institutions, Baltimore, Maryland; ³Department of Chemical and Biomolecular Engineering, Physical Sciences-Oncology Center, and Institute for NanoBioTechnology, Johns Hopkins University, Baltimore, Maryland; ⁴Department of Gynecology and Obstetrics, Johns Hopkins Medical Institutions, Baltimore, Maryland

High grade serous carcinoma is the most common and lethal type of ovarian cancer. Most patients are diagnosed at advanced stages and require the first-line therapy, which involves cytoreductive surgery followed by combined carboplatin and paclitaxel. While patients generally respond to the treatment at the beginning, only small percentages (10-15%) with advanced disease achieve long-term remission due to tumor recurrence. In this study, we demonstrate Spleen Tyrosine Kinase (SYK) as a new marker associated with recurrent post-chemotherapy high grade serous ovarian carcinomas. SYK is a non-receptor tyrosine kinase, first identified in activated B lymphocytes. Our results demonstrate that SYK inhibitors show a synergistic cytotoxicity with microtubule targeting agents including paclitaxel. Additionally, paclitaxel resistant cancer cells can be sensitized by co-treatment of paclitaxel and SYK inhibitor. Proteomic phosphotyrosine analysis reveals that SYK signaling participates in microtubule dynamics, and regulates the phosphorylation of tubulin and microtubule-associated proteins (MAP1B and MAP4). Furthermore, inhibition of SYK enhanced paclitaxel sensitivity through stabilizing microtubule in paclitaxel resistant tumor cells. Thus, targeting SYK pathway promises a new strategy to enhance paclitaxel response, especially in resistant ovarian cancers.

#9. Differential PARP inhibitors' effects on cell cycle and consequently homologous DNA damage repair

Petar Jelinic and Douglas Levine
Gynecology Research Laboratory, Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, NY, USA

Background: Poly (ADP)---ribose polymerase (PARP) inhibition has been explored in pre---clinical and clinical studies with greatest successes demonstrated in homologous DNA

damage repair (HDR) pathway--deficient cancer patients. PARP inhibitor's (PARPi) potency to inhibit PARP's catalytic activity is well established; however non--catalytic effects that may add to their therapeutic properties are not fully understood. Our objective was to comparatively evaluate non--catalytic effects of olaparib and veliparib, the two most widely used PARPi in clinical trials, with a particular focus on cell cycle effects and HDR.

Methods: To measure DNA damage repair activity upon PARPi treatment, we used a well established DR-GFP reporter assay in two model cell lines, U2OS^{DR--GFP} and H1299^{DR--GFP}. Cell cycle progression was assessed by propidium iodide and monitored by flow cytometry. Depletion of targeted mRNAs was performed by standard siRNA knock--down technique using electroporation for transfection. Immunoblotting was used to confirm cell cycle results and demonstrate replicative stress on the protein expression levels.

Results: We found a dramatic decrease in DNA damage repair activity in olaparib versus veliparib treated U2OS^{DR--GFP} p53 wild type cells, regardless of inhibitory potency. This decrease in HDR activity was a result of olaparib triggering a cell cycle arrest--like effect as a response to strong replicative stress. Lack of the same effect in p53-null cells suggests that olaparib's effect is p53-dependent, which was confirmed in U2OS^{DR-GFP} cells depleted of p53. Finally, CHK1 is strongly phosphorylated in cells treated with olaparib but not veliparib, confirming cells differential response to these two PARPi.

Conclusion: These data demonstrate that olaparib and veliparib differ in their non-catalytic inhibitory effects, with olaparib but not veliparib mitigating DNA damage repair activity via cell cycle arrest-like effects in a p53-WT background. Moreover, olaparib but not veliparib triggers CHK1 phosphorylation, which suggests differential levels of replicative stress. This new insight into olaparib's and veliparib's mechanism of action should be considered when developing inclusion criteria for treatment of cancer patients with these PARP inhibitors.

#10. Gene expression profiles of high--grade serous ovarian cancers in patients with normal CA125

Vance Broach, Fanny Dao, Douglas A. Levine

Gynecology Research Laboratory, Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, NY, USA

Objective: CA125 is a useful tool for monitoring response to therapy and recurrence in patients with high grade serous ovarian cancer (HGSOC). However, 15 to 20% of patients will have normal levels of CA125 at the time of recurrence, limiting the prognostic ability of CA125 in this population. We aimed to determine differences in gene expression profiles of tumors from patients with and without elevated CA125 levels at the time of recurrence.

Methods: Patients with stage IIIC or IV, HGSOC who underwent primary surgical cytoreduction and initial platinum--based chemotherapy were identified from The Cancer Genome Atlas (TCGA). All patients had platinum--sensitive, radiographic--confirmed recurrent disease. Patients were stratified into two groups; those with elevated CA125 (>35 U/mL) and those with normal CA125 (\leq 35 U/mL) at time of recurrence. Gene expression profiling was performed using one Agilent and two Affymetrix expression microarray platforms. Expression data was combined across microarrays using factor analysis for genes present on all array platforms. Gene expression levels for 11,864 genes were analyzed. Mean gene expression was compared using student t--test and significance was defined as $P < 0.001$.

Results: Data was available for 21 patients with normal (\leq 35 U/mL) CA125 and 32 patients with elevated CA125 (>35 u/mL) at the time of recurrence. There were no statistical differences in mean age (56 yrs, STD 11 yrs) or median time to recurrence (13 mos, range 6--50 mos) between the two study groups. Of the 11,864 genes analyzed, 14 showed a statistically significant difference in expression between the two groups (Figure); 6 genes had increased expression and 8 genes had decreased expression in patients with normal CA--125 levels at recurrence. The gene functions were consistent with known CA125/MUC16 biology including regulation of transcription and DNA binding, which would affect growth, mobility and invasion.

Conclusions: Gene expression profiles vary between advanced--stage, platinum--sensitive HGSOC patients who recur with and without elevated CA125 levels. These findings may indicate inherent differences in tumor biology. Further evaluation of the functional significance of this variable gene expression may lead to better stratification and monitoring approaches based on primary tumor genomics.

#11. PD--0332991, a cyclin--dependent kinase 4/6 inhibitor, is an active agent in uterine cancer cells

Jennifer A. Ducie, Petar Jelinic, Zichao Zhang, Fanny Dao, Maria Bisogna, Vicky Makker, Douglas A. Levine

Gynecology Research Laboratory,
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Objective: Uterine cancer is the most common gynecologic malignancy and has limited therapeutic options in the recurrent setting. PD-0332991 is a potent, orally active cyclin-dependent kinase (CDK) 4/6 inhibitor currently under investigation in many clinical trials. The best responses to PD--0332991 have been identified in retinoblastoma (*RB1*) wild-type cells. The Cancer Genome Atlas data on endometrial cancer indicate that 90% of tumors are *RB1* wild--type suggesting therapeutic applicability. We aim to establish benchmark sensitivities of uterine cancer cells to PD-0332991.

Methods: We measured the effects of PD--0332991 on proliferation and RB1 phosphorylation in a panel of six uterine cancer cell lines. Well--characterized *RB1* proficient and mutant cancer cell lines served as positive and negative controls, respectively. XTT Cell Proliferation assays were performed to assess drug sensitivity. Immunoblotting was performed to evaluate protein expression of total RB1, phosphorylated RB1, and CDKN2A (p16).

Results: The majority of uterine cancer cells tested was sensitive to PD--0332991. Leiomyosarcoma cell lines were the most sensitive to PD--0332991 with IC₅₀ values of ~ 0.25μM. Endometrial cancer cell lines had more variable response to PD--0332991 with IC₅₀ value ranging from 1 to 4μM. Decreased phospho--RB1 and increased p16, detected by immunoblotting, corresponded to the drug's inhibitory effect on proliferation.

Conclusions: PD-0332991 has an anti-proliferative effect in uterine cancer cell lines. As expected, most *RB1*--proficient cell lines were sensitive to the drug, with leiomyosarcomas being more sensitive than endometrial cancer cell lines. Data from our preliminary experiments suggest that PD-0332991 could be an active agent in the treatment of uterine cancer. Ongoing studies will determine if these effects are cell cycle dependent leading to a G1/S phase arrest and result in over-expression of *RB1*-dependent downstream effectors.

#12. The relationship between serous tubal intraepithelial carcinoma and invasive pelvic serous carcinoma

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Background: All high--grade serous carcinomas (HGSC) of the pelvis likely originate in the fimbriated portion of the fallopian tube. Serous tubal intraepithelial carcinoma (STIC) lesions are the putative precursor to HGSC.

Objective: Determine the frequency of the STICs in patients with advanced stage pelvic HGSC and evaluate whether the presence of a STIC lesion is associated with different clinical manifestations and / or outcome compared to those patients in whom a STIC was not identified.

Methods: All patients newly diagnosed with carcinoma of the fallopian tube, ovary, or peritoneum between 2009-2012 at Memorial Sloan-Kettering Cancer Center (MSKCC) were identified from hospital databases. Demographic, surgical, histopathologic, adjuvant therapy, follow-up and vital status data were collected retrospectively from electronic medical records. Inclusion criteria included high--grade (grade 3), stage IIIC or IV, serous histology, primary cytoreductive surgery at MSKCC. In cases that omitted any comment about the presence or absence of a STIC lesion in the pathologic specimen, all tumor-bearing tissue blocks were retrieved and corresponding slides were re-reviewed by a gynecologic pathologist. Statistical analyses were performed using IBM SPSS Statistics v21. Standard two--sided statistical tests were used as appropriate, including measures of association for categorical variables and time-dependent statistics for outcome metrics.

Results: Three hundred ninety eight women with fallopian tube, ovarian, or primary peritoneal carcinoma were identified between 2009--2012 at our institution. Two--hundred twenty--eight (57%) patients met all inclusion criteria. Median age of the population was 62.4 years (range, 32.2--86.9). Ninety--three (41%) cases had STIC lesions and 135 (59%) did not. Thirty--nine patients in this cohort had BRCA germline mutations; 16 (32.7%) in patients with STIC lesions and 23 (31.1%) in patients without

STIC. The majority of patients had stage IIIC disease (168, 73.7%) and received an optimal cytoreduction at time of their primary operation (207, 90.8%). Platinum---resistant disease was noted in 35 (17.1%) patients; 18 (21.4%) patients with STIC, and 17 (14.0%) in patients without STIC. Median follow-up among all patients in study was 26.9 months (range, 0.3-52.7). Of those with STIC lesions, 70 (75.3%) were reported in the original pathology report; 23 (24.7%) were identified upon study review. Of the patients without STIC, 25 (19%) had comprehensive tubal processing (SEE-FIM), 31 (23%) had representative tubal sections, 8 (6%) were tubal carcinomas without STIC lesions, and 79 (59%) are pending review.

After pathologic re-review of cases that did not report or had not originally identified STIC lesions, an additional 23 (24.7%) cases with STIC lesions were identified. The number of primary fallopian tube carcinoma diagnoses doubled from 61 (26.8%) to 119 (52.4%) after pathology re-review due to the identification of these additional cases with STIC. Kaplan---Meier survival curves did not demonstrate a statistical difference in progression-free (PFS) and overall-survival (OS) when comparing cases with and without STIC lesions ($P=0.11$ and $P=0.37$, respectively). However, a trend of poorer PFS among patients with STIC lesions was observed.

Conclusions: STIC lesions are present in approximately 41% of cases with HGSC of pelvic origin at our single institution. There are no differences in standard clinical parameters between STIC and non---STIC cases. There is a non---statistical increase in platinum---resistance and a corresponding decrement in progression---free survival for cases with STIC lesions. Further data is required to determine if this is a significant finding.

#13 The role of BRCA and CEBPD in serous ovarian cancer carcinogenesis

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Introduction: The most common histotype representing 90% of ovarian cancer cases is High Grade Serous Carcinoma. In a previous publication we found C/EBP-d to be significantly increased in fallopian tube epithelial in BRCA1 mutation carriers and specifically in the luteal phase of the menstrual

cycle, by transcription and protein expression. C/EBP-d represents a master regulator of gene transcription primarily through STAT3 activation in many tumor types. C/EBP-d is commonly induced by stress and is associated with increased DNA damage. Additionally, studies in breast cancer showed that C/EBP-d could interact with FANCD2, which is involved in DNA repair along with BRCA1 and BRCA2. C/EBP-d plays a dichotomous role in tumor suppression and promotion, which is tissue specific. We therefore studied the 1) differential expression of C/EBP-d in serous carcinoma 2) expression of C/EBP-d in precursor lesions (STIC) and 3) modeled the effect of over-expression of C/EBP-d in an *in vitro* fallopian tube cell culture model.

Methods: The study protocol for collection of tissue and clinical information for all patients was approved by the UHN REB. Tissue microarrays containing the different histotypes were used to validate immunohistochemical protein expression. Fallopian tubes with lesions classified as STIC using published diagnostic criteria, were selected (n=15) from women undergoing surgery for HGSC. FTE Cells were propagated on collagen IV coated plates in MCDB105/M199 media supplemented with BPE, EGF, insulin and hydrocortisone. Cells were immortalized via double infection with a lentiviral dominant negative TP53 vector and retroviruses with E7 and hTERT. Statistical analysis was performed using ANOVA ($p<0.05$) and Fisher's Exact Test ($p<0.05$).

Results/Conclusion: C/EBP-d is down regulated in over 60% of HGSC analyzed, an observation which is also histotype specific as LGSC tumors show high expression levels ($p<0.01$). 60% of C/EBP-d protein expression exhibited a gradual decrease from the normal FTE to the p53-signatures and the STICs and lost in concomitant HGSC. This indicates an inverse correlation between C/EBP-d and proliferative index. This suggests that C/EBP-d regulates cell-cycle progression in the FTE possibly through the G2/M checkpoint and is a potential tumor suppressor in HGSC. In short-term cultures of FTE cell lines, over-expression of C/EBP-d did not affect proliferation but may affect long-term growth. High expression of C/EBP-d in FTE cells correlated with decreased vimentin, N-cadherin, ACTA2 and Zeb2 while and in Twist1 and mir21 expression. This data is consistent with the phenotypic effect observed by over-expressing C/EBP-d in cell culture and indicates an involvement of C/EBP-d in mesenchymal-to-epithelial transition during high-grade serous carcinogenesis.

The role of BRCA and CEBPD in serous ovarian cancer carcinogenesis

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Introduction: The most common histotype representing 90% of ovarian cancer cases is High Grade Serous Carcinoma. In a previous publication we found C/EBP- δ to be significantly increased in fallopian tube epithelial in BRCA1 mutation carriers and specifically in the luteal phase of the menstrual cycle, by transcription and protein expression. C/EBP- δ represents a master regulator of gene transcription primarily through STAT3 activation in many tumor types. C/EBP- δ is commonly induced by stress and is associated with increased DNA damage. Additionally, studies in breast cancer showed that C/EBP- δ could interact with FANCD2, which is involved in DNA repair along with BRCA1 and BRCA2. C/EBP- δ plays a dichotomous role in tumor suppression and promotion, which is tissue specific. We therefore studied the 1) differential expression of C/EBP- δ in serous carcinoma 2) expression of C/EBP- δ in precursor lesions (STIC) and 3) modeled the effect of over-expression of C/EBP- δ in an *in vitro* fallopian tube cell culture model.

Methods: The study protocol for collection of tissue and clinical information for all patients was approved by the UHN REB. Tissue microarrays containing the different histotypes were used to validate immunohistochemical protein expression. Fallopian tubes with lesions classified as STIC using published diagnostic criteria, were selected (n=15) from women undergoing surgery for HGSC. FTE Cells were propagated on collagen IV coated plates in MCDB105/M199 media supplemented with BPE, EGF, insulin and hydrocortisone. Cells were immortalized via double infection with a lentiviral dominant negative TP53 vector and retroviruses with E7 and hTERT. Statistical analysis was performed using ANOVA (p<0.05) and Fisher's Exact Test p<0.05).

Results/Conclusion: C/EBP- δ is down-regulated in over 60% of HGSC analyzed, an observation which is also histotype specific as LGSC tumors show high expression levels (p<0.01). 60% of C/EBP- δ protein expression exhibited a gradual decrease from the normal FTE to the p53-signatures and the STICs and lost in concomitant HGSC. This indicates an inverse correlation between C/EBP- δ and proliferative index. This suggests that C/EBP- δ regulates cell-cycle progression in the FTE possibly through the G2/M checkpoint and is a potential tumor suppressor in HGSC. In short-term cultures of FTE cell lines, over-expression of C/EBP- δ did not affect proliferation but may affect long-term growth. High expression of C/EBP- δ in FTE cells correlated with decreased vimentin, N-cadherin, ACTA2 and Zeb2 while and in Twist1 and mir21 expression. This data is consistent with the phenotypic effect observed by over-expressing C/EBP- δ in cell culture and indicates an involvement of C/EBP- δ in mesenchymal-to-epithelial transition during high-grade serous carcinogenesis.

Fimbria and Ampulla Tubal Epithelium Have Similar Transcriptome Profiles

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Background: Recently described precursors of High Grade Serous Carcinoma, the p53 signature, a latent precursor, and Serous Tubal Intraepithelial Carcinoma, a pre-malignant precursor, occur most frequently at the distal and fimbriated end of the fallopian tube (FTE). We recently demonstrated that the FTE of BRCA1 mutation carriers, at genetic risk of HGSC, have altered signaling pathways compared to controls. A key question is whether the gene expression differences identified at the ampulla between BRCA1 and non- mutation carriers is similar to differences at the fimbria. This study determines the transcriptome profiles of normal fimbrial FTE and normal ampulla FTE which may lead to insight of why the distal end of the fallopian tube is preferentially predisposed to malignant transformation.

Method: Snap-frozen matched fimbria and ampulla tissues were controlled for age and ovarian cycle status. Cases included 12 luteal phase and 12 follicular phase women at no known risk for ovarian cancer. Laser capture microscopy was used to microdissect FTE cells, using 7-10 sections per case. Total RNA was isolated, RNA extracted and cDNA amplified. The expression profiles were generated using Affymetrix Human Genome HTA-2.0 Array.

Results: Using gene level differential expression analysis with the Affymetrix Expression Console software, we performed unsupervised hierarchical clustering analysis with all 24 samples. We used a fold change of < -2 or > 2 and ANOVA p-value < 0.05 as a cut-off criteria for selecting genes. The cases clustered predominantly by ovarian cycle status rather than by their differences in anatomical origin or their matched pair. There were 427 genes differentially expressed amongst the 4 groups – Fim-Luteal, Fim-Follicular, Amp-Luteal and Amp-Follicular.

Independent of ovarian cycle status, very few differences (35 genes – SALL1, SERPINA3, ANXA13, PDK4, ME1, GSTA1, GSTA2 – genes involved in metabolic pathways) were observed between the ampulla and fimbria FTE.

Conclusions: The epithelia of the anatomically high-risk fallopian tube – the fimbria, show few differences in gene expression profiles compared to the lower risk portion – the ampulla. Expression differences predominantly are in response to the hormonal milieu, i.e. the secretory and proliferative phases of the ovarian cycle. The increased anatomic risk of the fimbria is likely due to effects of the microenvironment, such as repeated exposure to follicular fluid at ovulation, rather than intrinsic differences of the FTE in the two sites.

THE FAMILIAL OVARIAN TUMOR STUDY: A MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL REVIEW

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Background: High grade serous carcinoma (HGSC) is associated with germline mutations of BRCA1/2 in approximately 15% of cases. The aim was to determine the accuracy of diagnostic classification of ovarian carcinoma and to determine if morphological and immunohistochemical (IHC) features distinguish HGSC with germline mutations from sporadic HGSC.

Design: Mutation screening and family pedigrees were obtained in 1414 women diagnosed from 1995 to 1999 and from 2002 to 2004. Representative tumor blocks were available for 645 cases. Tissue microarrays were stained for p53, WT-1, CK7, CK20, CDX2, HNF-1, PTEN. Reviewers were blinded to original diagnosis and to germline mutation status. Included in this study are 65 patients with documented BRCA mutations and a control group of HGSC patients (n=54) with both negative family history and negative germline testing results.

Results: A significant number of cases received a revised diagnosis after a review of morphology and IHC (n=128/645, 20%). The most frequently changed original diagnoses were endometrioid carcinoma (n=46/128, 36%), and adenocarcinoma NOS (n=19/128, 15%) - 89% were reclassified as HGSC. 15/128 (12%) cases originally diagnosed as invasive adenocarcinoma were revised to borderline tumor. Metastatic adenocarcinoma from the gastrointestinal tract was misclassified as a primary ovarian endometrioid or mucinous adenocarcinoma (n=5/128, 4%). Cases from community hospitals were more likely to have their diagnosis changed compared to those from academic centres (p=0.002). All patients with BRCA germline mutations had High Grade Serous Carcinoma. A morphological review showed that tumor infiltrating lymphocytes (TILs) were more prominent in the tumours of patients with BRCA mutations (p=0.02) compared to the Control group of sporadic HGSC. However, there was no significant difference in tumor grade, architecture, atypia, mitotic count, frequency of giant bizarre nuclei, presence of geographic necrosis, or IHC staining between HGSC patients with and without BRCA germline mutations.

Conclusion: IHC studies and review by an experienced gynecological pathologist are critical to correctly classify ovarian malignancies. Diagnoses are more likely to be revised in cases from community hospitals. The quantity of TILs is the only morphological feature that significantly differentiates BRCA germline mutation HGSC from sporadic HGSC. Biomarkers currently commonly used do not differentiate BRCA associated HGSC.

Tumor infiltrating lymphocytes (TILs) as a function of histological subtype and genetic background of ovarian epithelial carcinomas.

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Background

Ovarian epithelial carcinoma (OEC) comprises a large family of tumors with distinct clinicopathological, morphological, immunohistochemical, molecular, and genetic subtypes. Studies suggest that immune response and the presence of tumor infiltrating lymphocytes (TILs) are associated with longer disease free progression and survival. Furthermore, the presence of TILs can be correlated with the genetic background of the carcinoma, e.g. loss of BRCA1 expression. In this study, we sought to comprehensively examine a large set of various OECs for the presence of TILs as well as to determine any association with BRCA germline mutation status and loss of mismatch repair (MMR) protein expression.

Design

Formalin-fixed paraffin embedded tissues (n=678) from a wide variety of OECs were obtained as part of population-based familial ovarian cancer study. Known BRCA mutation carriers (n=65) and a Control group of HGSC cases with negative family history and germline testing (n=54) were included in this study. Using automated image analysis and manual scoring of tissue microarrays, the number of CD3+, CD8+, Foxp3+, CD68+ cells were enumerated. MMR protein status was determined by manual scoring of MLH1, PMS2, MSH2, and MSH6 immunohistochemistry. Statistical analysis was performed using ANOVA ($p < 0.05$) and Fisher's Exact Test ($p < 0.05$).

Results

The frequency of TILs did not statistically differ between different subtypes of OEC ($p = 0.66$ to $p = 0.85$). Morphologically, increased number of TILs was associated with loss of MMR protein expression in endometrioid OEC ($p < 0.02$) as well as BRCA+ high grade serous carcinoma (HGSC) ($p < 0.005$). Loss of MMR protein expression was found to be more frequent in endometrioid OEC and intact in all serous and mucinous borderline tumors. CD68+ macrophages were found more frequently in BRCA+ HGSC ($p < 0.045$).

Conclusion

The frequency of TILs and CD68+ macrophages varies amongst different morphological subtypes of OEC and genetic background. This data may provide further insight into the interplay of morphological features, immune response, and genetic factors in determining clinical behavior and patient outcome.

Hormone Receptor Status Predicts Clinical Outcome of High Grade Serous Carcinoma: A Gene Expression Study

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Background: High Grade Serous Carcinoma (HGSC) originates in the hormonally sensitive fallopian tube epithelium. Most HGSC cases express estrogen receptor (ER). Anti-estrogen therapy is often used to treat recurrent disease, but associations between ER expression and tumor response are not well documented. We hypothesize that i) reproductive hormone signaling is altered in HGSC, that ii) reproductive hormone receptor status (ER α /PR) may be an important indicator of clinical outcome and that iii) ER target gene expression will provide further insight into the use of anti-hormone therapy in ovarian cancer.

Design: FFPE tissues (n=334) and snap-frozen tissues (N=43) from HGSC naïve to chemotherapy were obtained from the UHN Pathology Department. Clinical outcome data inclusive of both recurrence-free (RFS) and overall survival (OS) was available on 165 cases. Using tissue microarrays and standard immunohistochemistry, ER α and PR expression was analyzed using automated image analysis. A candidate list of ER target genes was generated by comparing tumor gene expression profiles of the sub-groups based on ER/PR expression. Candidate genes were validated by qPCR and immunohistochemistry on tissue microarrays. Statistical analysis was performed using ANOVA (p<0.05) and Fisher's Exact Test (p<0.05).

Results: High PR expression cases (31%) had a significant increase in both RFS (p<0.05) and OS (p<0.005) compared to the PR-low samples. ER expression, positive in 88% of cases, did not predict for survival. Combining expression of PR with ER created four subgroups, with ER-high/ PR-low being the most frequent expression pattern (57%), followed by ER-high/ PR-high (25%), ER-high/ PR-low (12%) and ER-low/PR-high (5%). Overall survival was prolonged in the ER-low/PR-high cases with the shortest OS seen in the ER-high/PR-low patients (p=0.002). Gene expression analysis revealed 202 ER target genes with more than a 2 fold-change in expression (p<0.05). Five genes (DKK3, SOX11, IGF2, TKTL1, NY-ESO-1) were selected for validation by qPCR and IHC, based on gene ontology and cancer pathway associations.

Conclusions: HGSC is predominantly an ER-high/PR-low cancer, but other patterns of hormone receptor expression exist which predict for an increased overall survival. ER-high differ transcriptionally from ER-low HGSC although some known ER target genes are still transcriptionally activated in the absence of PR. This information should provide further insight into the use of hormone receptors as indicators of anti-hormone therapy response in ovarian cancer.

Origins of ovarian cancer, the biology of pre-malignancy, and cancer health disparities

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Epithelial ovarian cancer (EOC) is the 5th leading cause of cancer-related deaths in women in the United States¹. High-grade serous carcinoma (HGSC) is the most aggressive EOC and women usually present at high-grade and high stage, leading to only a 30% 5 year survival rate after adjuvant platinum-based chemotherapy². Women who have an inherited mutation in the BRCA1 or BRCA2 genes have a substantial increased lifetime risk (11%-60%) in developing high-grade serous cancer – the predominant histotype associated with hereditary breast-ovarian cancer³. The distal end of the fallopian tube is now the accepted site of origin of high-grade serous ovarian cancer⁴. It is known that factors related to parity, ovulation and hormone regulation have a dramatic effect on the risk of developing ovarian cancer in both BRCA mutation carriers and non-carriers. Recently, we have shown that the transcriptional profile of histologically normal fallopian tube epithelia of BRCA mutation carriers is different from wild type FTE and possibly hints at BRCA haploinsufficiency^{5,6}. We propose that changes of the transcriptome in BRCA mutation carriers reflect an altered response to the microenvironment ovulatory stresses, which may include altered reproductive hormone levels and the ovulatory inflammatory response.

The prevalence of the BRCA mutation in the Bahamas where 23% of unselected breast cancer patients carry a mutation in the BRCA1 is the highest hereditary proportion in the world^{7,8}. We are determining the importance of genetic founder effects in the two BRCA genes in the

Caribbean basin - a region with a large African population (the Caymans, Barbados, Dominica and Jamaica). Epithelial ovarian cancer rates are low in the Caribbean and risk factors such as parity, age at menarche and menopause may be strong indicators of decreased risk in the population. The results of this extensive study will help direct health policies towards genetic testing in the Caribbean with implications for the immigrant US population, and should lead to regional policies regarding preventative strategies.

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Retinoblastoma pathway deregulatory mechanisms determine clinical outcome in high-grade serous ovarian carcinoma

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Alterations in the retinoblastoma pathway are frequent in ovarian/tubal high-grade serous cancers, but the mechanism of deregulation and the impact on patient outcome are poorly understood. A cohort of 334 high-grade serous carcinomas was studied by immunohistochemical analysis of RB1, p16, cyclin D1, cyclin E1, and Ki67. Additional detailed analyses including *RB1* allelic deletion ($n=42$), mutation ($n=75$), methylation ($n=31$), and SNP array analyses ($n=75$) were performed on cases with clinical parameters, including age, debulking status, treatment, and clinical outcome. p16/RB1 expression results yielded three distinct clinically relevant subgroups upon multivariable analysis controlling for stage, debulking status, and treatment types: p16 homogeneous/RB1 + with the shortest progression-free survival (median 15 months (95% CI: 13–18); $P=0.016$) compared with the p16 heterogeneous/RB1 + subgroup (median 22 months (95% CI: 16–32)) and the p16 homogeneous/RB1 – subgroup (median 20 months (95% CI: 15–24)). Patients in the p16 homo/RB1 – subgroup showed a significant increase in overall survival (>60 months; $P=0.013$), which suggests an increase in sensitivity to cytotoxic agents. Analyses of Rb pathway mechanistic differences among these groups revealed frequent *RB1* genomic alterations such as *RB1* allelic loss and/or large spanning deletions (83%) in the p16 homo/RB1 – subgroups, also indicating that *RB1* deletions are frequent in high-grade serous carcinoma. *CCNE1* gene gains/amplifications were frequent in the p16 homogeneous/RB1 + subgroup (68%) and cyclin D1 protein overexpression was predominantly characteristic of the p16 heterogeneous/RB1 + subgroup. These subcategories occur early in tumor progression and are seen with similar frequency in the cancer precursor lesion, serous tubal intra-epithelial carcinoma. Overall, this study uniquely identifies multiple non-synonymous mechanisms of retinoblastoma pathway deregulation that correlate with significantly different clinical outcomes. Furthermore, deregulations identified in precursor lesions suggest a key role of this pathway in serous tumor development. Recognition of these categories may identify patients with increased sensitivity to chemotherapy and new opportunities for novel therapeutics.

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Keywords: clinical outcome; genomic alterations; ovarian carcinoma; retinoblastoma pathway; serous tubal intra-epithelial lesions

High-grade serous cancer is the most common type of epithelial ovarian cancer, accounting for about 70% of all cases, and for the majority of epithelial ovarian cancer deaths.^{1–4} Despite an initial

high response rate to first-line adjuvant platinum-based chemotherapy and improvements in surgical and medical treatment options, the prognosis remains poor for most high-grade serous carcinoma patients.^{3,4} Key challenges in improving overall survival include limited understanding of the early events in serous carcinogenesis, a lack of cancer screening strategies leading to over 90% diagnosis at an advanced stage of disease and ineffective treatment options for recurrent, platinum-resistant disease.^{2–4} Recent advances in molecular

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pathogenesis by genomic analyses demonstrate that high-grade serous carcinoma has a strikingly high prevalence of somatic copy number alterations, with few recurrent mutations.^{3–5} The spectrum of mutations is distinct from other types of epithelial ovarian cancer, reflecting the differing etiology, pathogenesis, and cell of origin in the different histotypes. In addition, deregulation of various pathways, including retinoblastoma, PI3K/Ras, homologous recombination, and Notch signaling pathways, may be used to further stratify high-grade serous carcinoma into molecular subgroups with potential relevance for use of targeted therapies.^{5,6}

The retinoblastoma pathway is an important regulator of proliferation at the G1/S checkpoint, and disruption of at least one arm of the retinoblastoma pathway is frequent and required in tumor formation/progression.^{7–9} Differential genetic and epigenetic mechanisms of retinoblastoma pathway disruption may lead to non-synonymous consequences dependent on tissue and tumor type.^{7–10} Deregulation of key pathway members, such as cyclin D1 and cyclin E1 in breast carcinoma and RB1 and *CDKN2A* in lung carcinoma, predicts for clinical outcome and allows the differential alterations to be exploited when considering the efficacy of common and new therapeutic targets.^{7–10}

Aberrant *CDKN2A* elevation, identified by intense homogeneous expression, is seen in many cancers with loss of *RB1* function, which has thus far been demonstrated to enable bypass of *CDKN2A*-mediated cell cycle arrest.^{11,12} For example, p16 overexpression in cervical cancer is a surrogate of human papillomavirus infection (oncoprotein E7 targets RB1).^{13,14} Similarly, elevated p16 occurs in 70% of high-grade serous carcinoma, but the *RB1* mutation rate is low (4–6%) and only 6% of cases have protein loss.^{5,15} Furthermore, the correlation between RB1 and p16 protein expression in high-grade serous carcinoma has not yet been explored in depth and associations between different retinoblastoma pathway alterations and clinical outcome/treatment response remain poorly investigated. In this study, we identify non-synonymous retinoblastoma pathway deregulation modalities that stratify patients with different responses to first-line platinum-based chemotherapy.

Materials and methods

Tissue Samples

The study protocol for collection of tissue and clinical information for all patients was approved

by the UHN Research Ethics Board. All patients provided written informed consent authorizing collection and use of tissue for research purposes. Snap-frozen ($n=75$) and formalin-fixed paraffin-embedded ($n=334$) tissues were retrospectively selected from the University Health Network Biobank (1996–2008). Key inclusion criteria were high-grade serous tumors from patients naive to chemotherapy. Clinical annotation inclusive of age at diagnosis, stage, surgical debulking status, first-line chemotherapy, progression-free and overall survival was available for 210 cases (Supplementary Table 1). Progression-free survival was defined as the time elapsed between diagnosis and progression, based on the first confirmed sign of disease recurrence. Overall survival was defined as the time elapsed between diagnosis and death by disease or date of last follow-up.

Immunohistochemistry

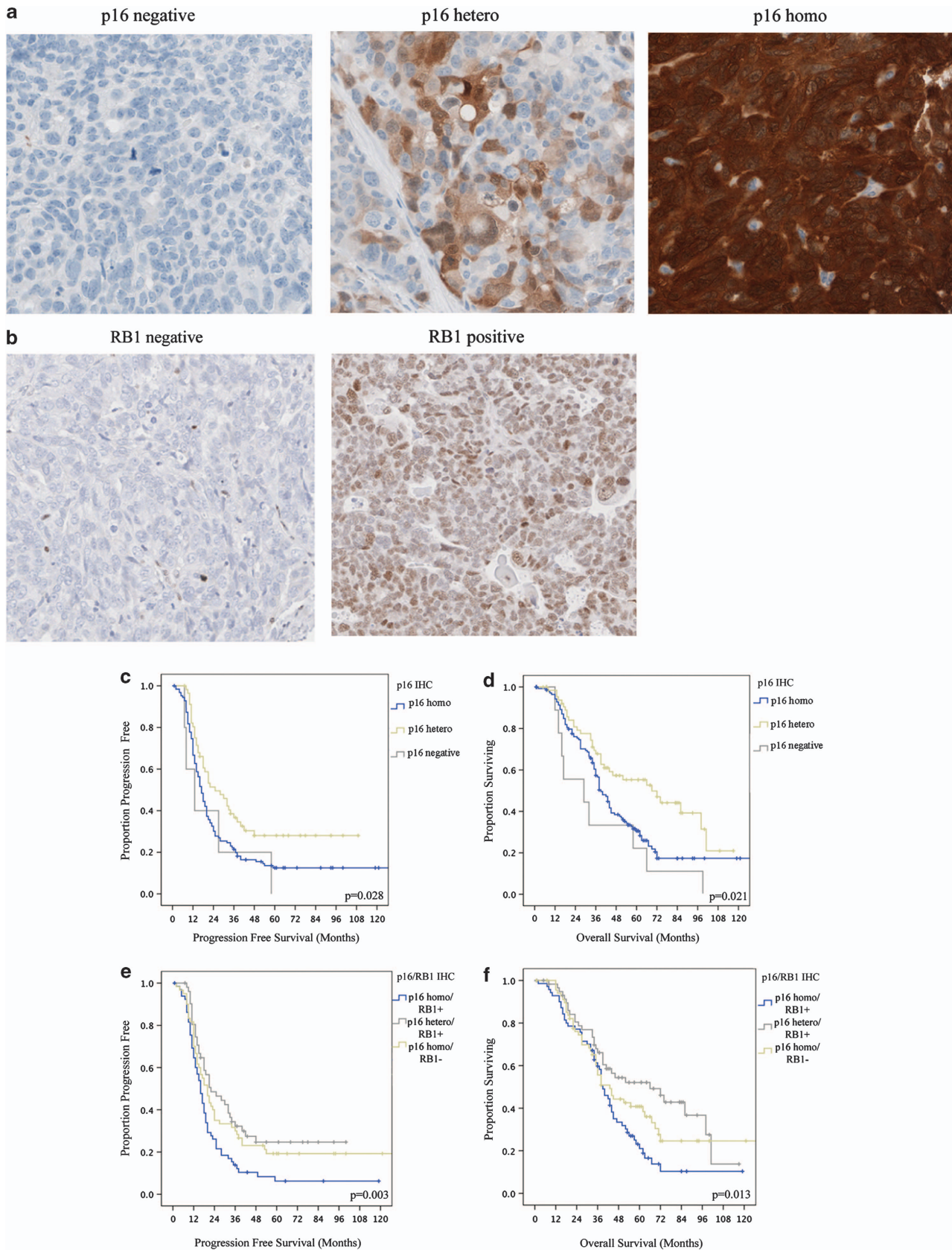
Protein expression for p16 (CINtec® Histology V-Kit, Roche mtm Laboratories) and RB1 (NCL-RB1, Novacastra) was assessed by immunohistochemistry using standard techniques on tissue microarrays composed of 334 high-grade serous carcinoma formalin-fixed paraffin-embedded tissues.

p16 expression was divided into three categories: 0 = negative, 1 = heterogeneous staining (patchy staining in three tumor fields), 3 = diffuse homogeneous staining (100% staining in three tumor fields; Figure 1a). RB1 protein expression was divided into a positive and negative stain (defined by complete absence of signal; Figure 1b). Protein expression for cyclin E1, cyclin D1, and Ki67 was also determined (Supplementary Table 2).

RB1 Allelic Loss, Mutation, Methylation, and Sequencing Analyses

These analyses were performed in collaboration with *Impact Genetics*, a clinical testing laboratory that has optimized molecular assays to identify *RB1* mutations. Patient genomic DNA was isolated from normal fallopian tube/ovarian and high-grade serous carcinoma tumor tissues using the DNeasy kit (Qiagen, Canada). Quantitative multiplex PCR was performed on 75 patient samples to detect deletions, duplications, or insertions, and exon copy number in exons 1–27 with flanking introns and the promoter region. Allele-specific PCR was performed to detect SNPs at 11 locations based on known recurrent nonsense *RB1* mutations and *RB1* promoter methylation status was determined on 31 tumors

Figure 1 Kaplan–Meier curves of the differences identified in progression-free survival and overall survival based on p16/RB1 immunohistochemical stratification. Representative images of immunohistochemical scoring for (a) p16—negative, heterogeneous, homogeneous; (b) RB1—negative and positive ($\times 20$ magnification). Patient subgroups with differential p16 expression patterns showed significant differences in progression-free survival and overall survival (c) and (d). Patient subgroups with differential p16/RB1 expression patterns also showed significant differences in progression-free survival and overall survival (e) and (f).



as previously described.^{16–18} For *RB1* allelic analyses ($n=42$), three polymorphic markers were used: a variable number of tandem repeats in intron 20 (RB1.2) and two microsatellite markers D13S153 and D13S164 (Supplementary Figure 1D). A sample marker was called ‘informative’ when two alleles with a different number of variable number of tandem repeats were present in the normal DNA. *RB1* allelic loss at a particular polymorphic region was determined based on the height differences of the cancer and normal control peaks using the allelic imbalance ratio (AIR) = $(T1) \times (N2) / (T2) \times (N1)$, from Banks *et al.*, where allelic loss = $AIR > 1.2$, $AIR < 0.8$.¹⁹

Statistical Analysis

Associations between the p16/RB1 subgroups and clinical outcome were examined using Kaplan–Meier survival analysis and log-rank test. Multivariable analyses for progression-free survival and overall survival were performed to control for debulking status, stage, and treatment using the Cox model. Associations between copy number/mutations/allelic loss alterations of Rb pathway members and the p16/RB1 subgroups were identified using Pearson χ^2 analyses. To determine differential expression of cyclin D1, cyclin E1, and Ki67 across the identified RB1/p16 subgroups, ANOVA and Tukey’s honest Range significance tests were performed. SPSS 14.0 (SPSS, Chicago, IL, USA) and SAS 9.2 were used to perform these analyses.

Results

p16/RB1 Protein Expression Patterns Define Clinical Outcome in High-Grade Serous Carcinoma

Immunohistochemical analyses for p16 and RB1 with three fields assessed per tumor were performed on a cohort of 334 high-grade serous carcinoma cases with a median follow-up time of 41 months (95% CI: 38–45). p16 expression was characterized by three patterns: negative (6%), heterogeneous (31%), and homogeneous (63%; Table 1, Figure 1a). Patients with heterogeneous expression showed a significantly longer progression-free survival (median 22 months (95% CI: 16–32)); $P=0.028$) and overall survival (median 66 months (95% CI: 38–86)); $P=0.021$) (Table 1, Figure 1c and d). RB1 immunohistochemistry identified two subgroups, negative (33%) and positive (67%; Table 1, Figure 1b). There was no significant difference between these groups in age, progression-free survival (median 20 vs 18 months, respectively; $P=0.339$), or overall survival (median 44 vs 42 months, respectively, $P=0.720$; Table 1).

Next, we analyzed the effects on clinical outcome of combining p16 and RB1 protein expression patterns for all high-grade serous carcinoma cases,

where six subgroups were analyzed, the most prevalent being: p16 homogeneous/ RB1 positive (p16 homo/RB1+) at 34%, p16 heterogeneous/RB1 positive (p16 hetero/RB1+) at 27% and p16 homogeneous/RB1 negative (p16 homo/RB1–) at 28% of all samples (Table 1). The median progression-free survival and overall survival for the poor outcome group (p16 homo/ RB1+) were 15 months (95% CI: 13–18) and 39 months (95% CI: 35–44; Table 1). Patients in the p16 homo/RB1+ subgroup showed a shorter progression-free survival compared with the p16 hetero/RB1+ and p16 homo/RB1– subgroup (median 22 vs 20 months, respectively, $P=0.003$; Table 1, Figure 1e). Likewise, the p16 homo/RB1+ subgroup showed a shorter overall survival compared with the p16 hetero/RB1+ and p16 homo/RB1– (median 66 vs 44 months, respectively, $P=0.013$; Table 1, Figure 1f). Following multivariable analyses, considering predictors of outcome, such as debulking status (optimal/sub-optimal), treatment (platinum vs platinum and taxane), and stage (low/high), significantly shorter progression-free survival ($P=0.016$) and overall survival ($P=0.049$) was found in the p16homo/RB1+ subgroup compared with the p16 hetero/RB1+ and the p16 homo/RB1– subgroups (Table 2).

RB1 Hemizygous Deletions Are Frequent in High-Grade Serous Carcinoma and Correlate with RB1 Functional Loss

Forty-two high-grade serous carcinoma tumors and their corresponding normal tissues were analyzed for *RB1* allelic deletions at three polymorphic regions (D13S153, RB1.20, D13S164) located on chromosome 13q14.2; thirty-eight samples were informative for at least one marker (Supplementary Figure 1D). Twenty-nine out of thirty-eight (76%) samples had allelic loss at either D13S153 or RB1.20 and fifteen out of twenty-nine (52%) samples correlate with a loss of RB1 protein expression (Figure 2a). Furthermore, 21/29 (72%) of samples with *RB1* allelic loss have p16 homogeneous expression (Figure 2a). These findings show that *RB1* allelic loss is prevalent in high-grade serous carcinoma and that it correlates with p16 overexpression.

The cohort of 75 high-grade serous carcinoma samples was utilized for a thorough investigation of the *RB1* gene, by performing copy number, mutation, and hypermethylation analyses. *RB1* hemizygous deletions were identified in 38% of samples, however, no homozygous deletions were detected by SNP analysis and *RB1* copy number gains were identified in only 6% samples (Supplementary Figure 1A and B). Functional loss associated with *RB1* hemizygous deletions, as 71% (20/28) of samples, showed downregulation of *RB1* mRNA ($P=0.0008$) and a loss of protein expression (Supplementary Figure 1C and Supplementary Table 3).

Table 1 Frequency of high-grade serous carcinoma cohort stratification based on p16/RB1 immunohistochemistry between the three prevalent RB1/P16 subgroups (p16 homo/RB1 +, p16 hetero/RB1 +, p16 homo/RB1 –) and the results of the univariable analyses and clinical characteristics such as age, debulking status, treatment, progression-free survival, and overall survival

<i>p16/RB1 Staining (All samples)</i>	<i>RB1 expression</i>			<i>p16 expression</i>				<i>p16/RB1 subgroups</i>			
	<i>Negative</i>	<i>Positive</i>	<i>P value</i>	<i>Negative</i>	<i>Hetero</i>	<i>Homo</i>	<i>P value</i>	<i>p16 Homo/ RB1 +</i>	<i>p16 Hetero/ RB1 +</i>	<i>p16 Homo/ RB1 –</i>	<i>P value</i>
Frequency % (n = 336)	112 (33%)	224 (67%)		21 (6%)	105 (31%)	210 (63%)		115 (34.2%)	92 (27.4%)	95 (28.3%)	
Median age, years (n = 334)	57	59	0.227	59	58	59	0.461	61	57	58	0.104
Optimally debulked patients/ total patients with debulking status data (n = 112/207)	54%	54%	0.554	56%	55%	53%	0.962	52%	56%	55%	0.998
Platinum + taxane-treated patients/total patients with treatment data (n = 188/210)	92%	90%	0.468	88%	88%	93%	0.511	93%	86%	92%	0.217
Progression-free survival median (95% CI) n = 206	20 (15–24)	18 (15–20)	0.339	22 (14–31)	22 (16–32)	17 (15–20)	0.028	15 (13–18)	22 (16–32)	20 (15–24)	0.003
Overall survival median (95%CI) n = 206	44 (35–64)	41 (37–50)	0.72	33 (25–41)	66 (38–86)	39 (36–45)	0.021	39 (35–44)	66 (38–86)	44 (35–64)	0.013

p16 hetero/RB1 +, p16 homo/RB1 –, and p16-/RB1 – were excluded due to small sample size.

Bold values represent statistically significant values.

Mutation analyses (quantitative multiplex-PCR and allele specific-PCR) revealed 40 different *RB1* gene alterations across the cohort: 30 exonic deletions, 1 homozygous single-base mutation, and 9 exonic amplifications (Figure 2c). Mapping of the deletions and amplifications onto RB1 protein domains did not reveal any particular mutation hotspots, as most span the entire gene region or are multi-exonic deletions (Figure 2c). In the p16 homo/RB1 – subgroup, 26/31 (83%) of samples resulted in functional loss of protein, including the homozygous mutation (R445X; Figure 2b and c). Strong synergy was found between SNP and mutation analyses in terms of RB1 deletions identified. Methylation analyses on all 31 p16 homo/RB1 – group samples revealed no promoter hypermethylation (data not shown).

p16/RB1 Subgroups Show Characteristic Deregulations of the Retinoblastoma Pathway

Copy number alterations were found in all seven key retinoblastoma pathway members with correlative changes in mRNA expression in five members: *RB1*, *CDKN2A*, *CCNE1*, *E2F3*, and *CDK4* (Supplementary Table 3 and Supplementary Figure 1B). *CCNE1* overexpression is a striking feature of p16 homo/RB1 +, with copy number gains and amplifications in 68% (13/19; $P=0.005$; Figure 3c) and showed significant protein overexpression compared with the other p16/RB1 subgroups ($P<0.05$; Figure 3a and b), suggesting that genomic gain/amplification of this oncogene has an important role in this subgroup. Another trend was also observed with *E2F3*, where all five amplifications and many of the copy number gains (10/29) were

identified in p16 homo/RB1 – subgroup samples ($P=0.08$).

Cyclin D1 immunohistochemistry on 334 samples revealed predominant protein overexpression in the p16 hetero/RB1 + subgroup ($P<0.05$; Figure 3a and b), although *CCND1* copy number alterations did not correlate with *CCND1* mRNA expression (Supplementary Table 3) or with any particular p16/RB1 subgroup (Figure 3d). In addition, cyclin D1 protein expression observed in the p16 hetero/RB1 + subgroup was significantly higher than in the normal fallopian tube epithelium (Supplementary Figure 3). These results suggest that cyclin D1 overexpression, does occur in high-grade serous carcinoma, is most frequent in the p16 hetero/RB1 + subgroup and most likely occurs as a result of post-translational modifications. Interestingly, the p16 hetero/RB1 + subgroup contained a large proportion of samples without any retinoblastoma pathway copy number alterations, indicating that patients in this cohort are largely retinoblastoma pathway neutral except for those with cyclin D1 overexpression.

We also analyzed Ki67 immunohistochemistry in the 334 high-grade serous carcinoma samples to estimate proliferative index. The p16 homo/RB1 – subgroup exhibited the highest Ki67 expression compared with either p16 hetero/RB1 + ($P<0.05$) or p16 homo/RB1 + subgroups ($P<0.05$; Figure 3a and b). Overall, high-grade serous carcinoma by definition are considered to have a high mitotic rate, but these results indicate the presence of a wide proliferative Ki67 spectrum (18–89%), with patients in the p16 hetero/RB1 + group at the lowest end (mean percent of Ki67 = 42.5%; Figure 3b). These analyses have revealed key features of retinoblastoma pathway deregulation across the clinically distinct groups: *CCNE1* gain/amplification in the

Table 2 Multivariable models for predicting progression-free survival and overall survival include all significant predictors: p16/RB1 immunohistochemistry groups, debulking status, and treatment, except age at diagnosis (not significant)

	Progression-free survival				Overall survival			
	Hazard ratio	P value	95% CI		Hazard ratio	P value	95% CI	
Age at diagnosis	—	0.951	—	—	—	0.29	—	—
Debulking (optimal vs suboptimal)	1.86	<0.001	1.32	2.61	2.36	<0.001	1.64	3.4
Treatment (platinum vs platinum + taxane)	1.9	0.03	1.06	3.39	4.17	<0.001	2.33	7.47
p16 hetero/RB1 + vs p16 homo/RB1 +	0.57	0.01	0.37	0.87	0.57	0.016	0.36	0.9
		0.016				0.0497		
p16 homo/RB1 – vs p16 homo/RB1 +	0.63	0.024	0.43	0.94	0.75	0.178	0.49	1.14

Bold values represent statistically significant *P*-values.

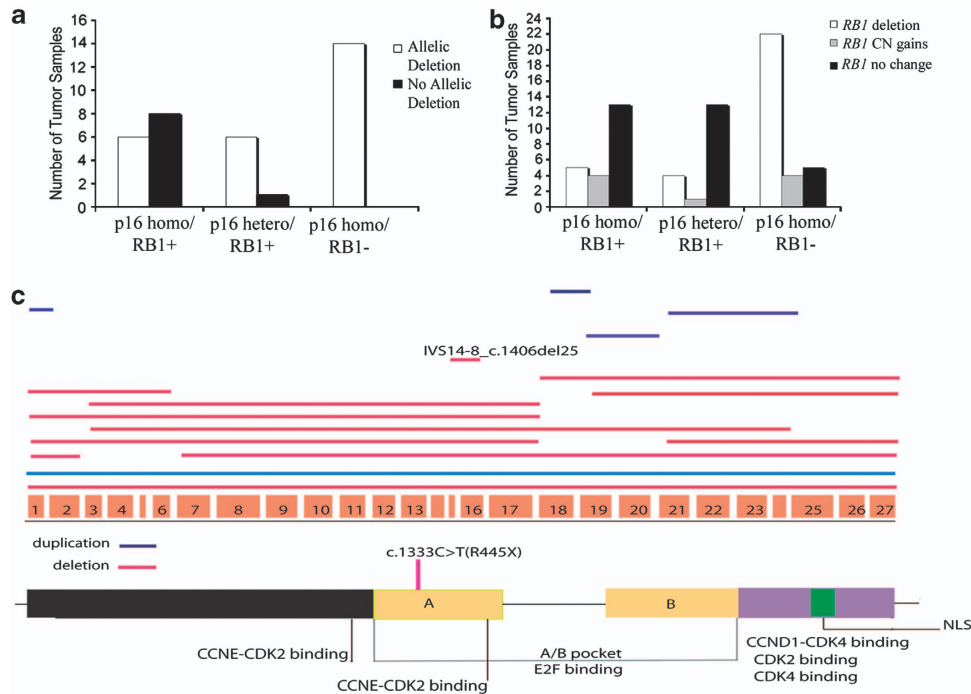


Figure 2 Deregulations at the *RB1* gene (chromosome 13q14.2). (a) Correlations between *RB1* allelic loss and the three clinically distinct p16/RB1 subgroups. (b) Correlations between *RB1* copy number alterations and the three clinically distinct p16/RB1 subgroups. (c) Summary of all *RB1* gene alterations illustrating a high frequency of whole gene or large spanning gene deletions and few point mutations.

p16 homo/RB1+ group, cyclin D1 overexpression in the p16 hetero/RB1+ group, and *RB1* genomic alterations along with a high-proliferative index in the p16 homo/RB1 – group.

p16/RB1 Deregulation Is an Early Event in High-Grade Serous Carcinoma Development

p16 and RB1 protein expression were analyzed by immunohistochemical analysis in the immediate

precursor of high-grade serous carcinoma—serous tubal intraepithelial carcinoma, as recently defined by morphologic and immunohistochemistry criteria (p53 and Ki67; Supplementary Figure 2).^{20–24} Histological normal fimbriae (used as controls) showed weak p16 expression with few cells staining positive and intermediate levels of RB1 expression with dispersed regions of weaker staining (Supplementary Figure 3). p16 homogeneous expression was found in 73% (11/15) of serous tubal intraepithelial carcinomas, the same

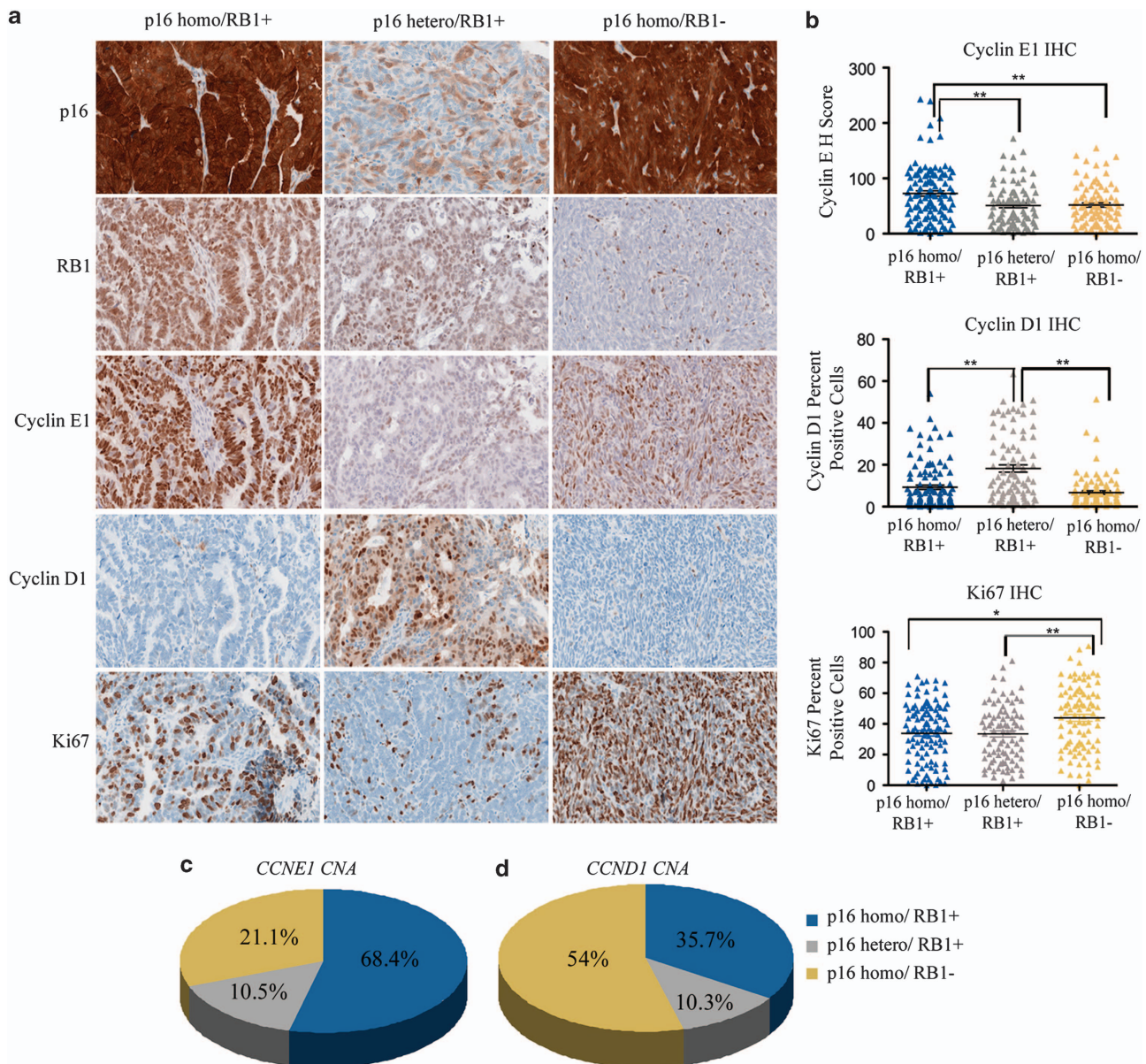


Figure 3 Immunohistochemical quantification of cyclin E1, cyclin D1, and Ki67 protein expression and quantification of *CCNE1* and *CCND1* CNA across the p16/RB1 clinical subgroups. (a) Immunohistochemical representation of cyclin E1, cyclin D1, and Ki67 (× 20 magnification). (b) Protein expression quantification of cyclin E1, cyclin D1, and Ki67; (c) copy number alterations (CNA) of *CCNE1* and (d) *CCND1* across the p16/RB1 subgroups (* $P < 0.05$, ** $P < 0.01$).

expression pattern as in their concomitant high-grade serous carcinoma, whereas only 20% (3/15) of these precursor lesions had p16 heterogeneous expression (Figure 4). The p16/RB1 subgroups identified in the established tumors were also found in serous tubal intraepithelial lesions at similar frequencies to the cancer: 47% of serous tubal intraepithelial carcinomas in the p16 homo/RB1+ subgroup, 27% p16 homo/RB1-, and 13% in the p16 hetero/RB1+ subgroup (Figure 4). Only one p16-/RB1+ serous tubal intraepithelial carcinoma was identified, a pattern shared with the corresponding high-grade serous carcinoma. This is in agreement with the high-grade serous carcinoma

tumor data, which only had 23/334 p16- samples, suggesting that p16 inactivation is infrequent in this disease. These data suggest that retinoblastoma pathway deregulation occurs early in high-grade serous carcinoma tumorigenesis.

Discussion

This study uses a large tumor cohort to comprehensively determine the different non-synonymous retinoblastoma pathway deregulations with relevance to patient outcome that predict for both progression-free survival as well as overall survival.

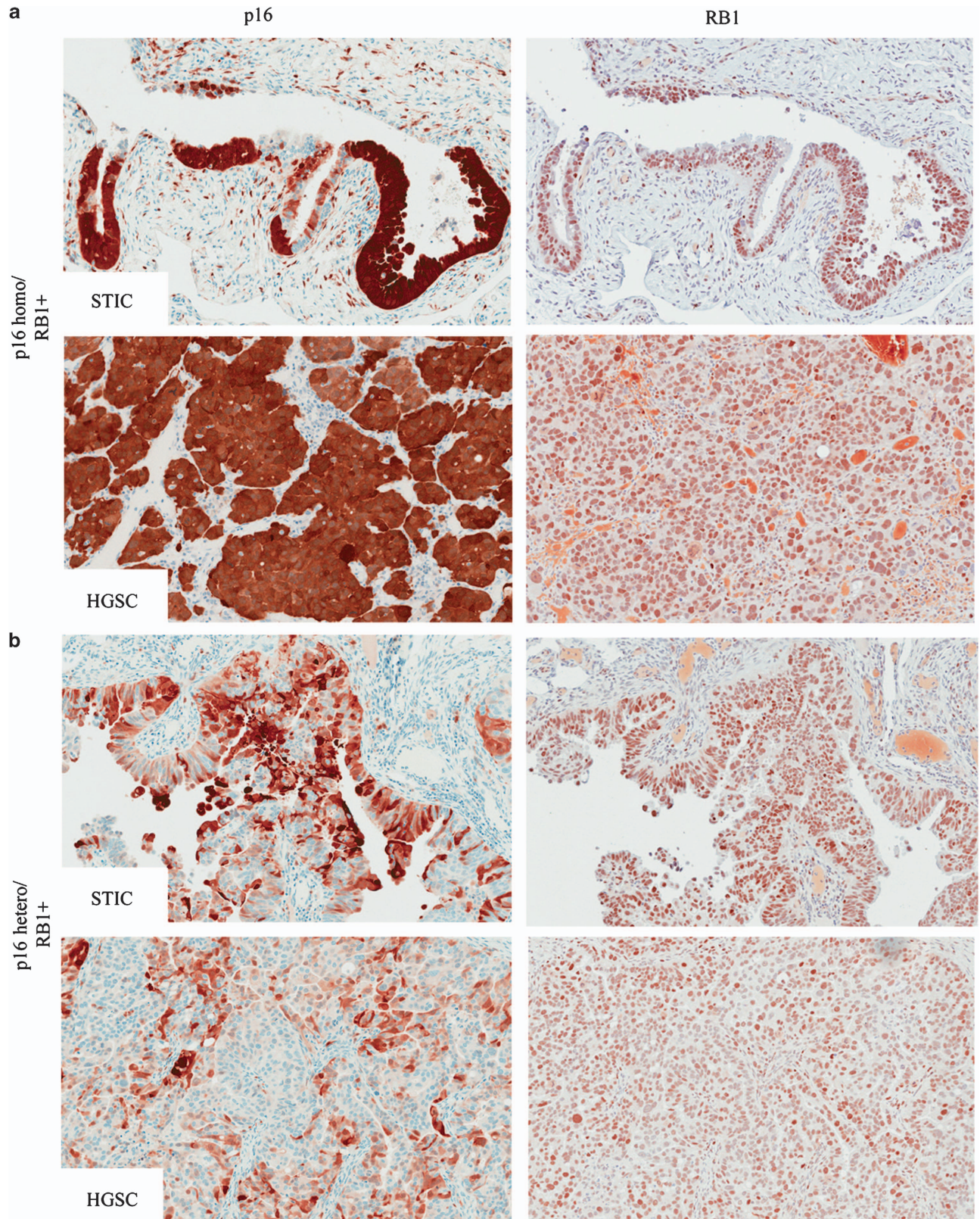


Figure 4 p16/RB1 immunohistochemistry patterns shared across serous tubal intraepithelial carcinomas and high-grade serous carcinoma ($\times 20$ magnification). **(a)** p16 homo/RB1+ subgroup identified in 47%. **(b)** p16 hetero/RB1+ subgroup identified in 13%. **(c)** p16 homo/RB1- subgroup identified in 27%. The prognostic signature identified in invasive cancers is also found in concomitant serous tubal intraepithelial carcinomas. The definition of serous tubal intraepithelial carcinoma includes morphological characteristics and immunohistochemical staining of Ki67 and p53.

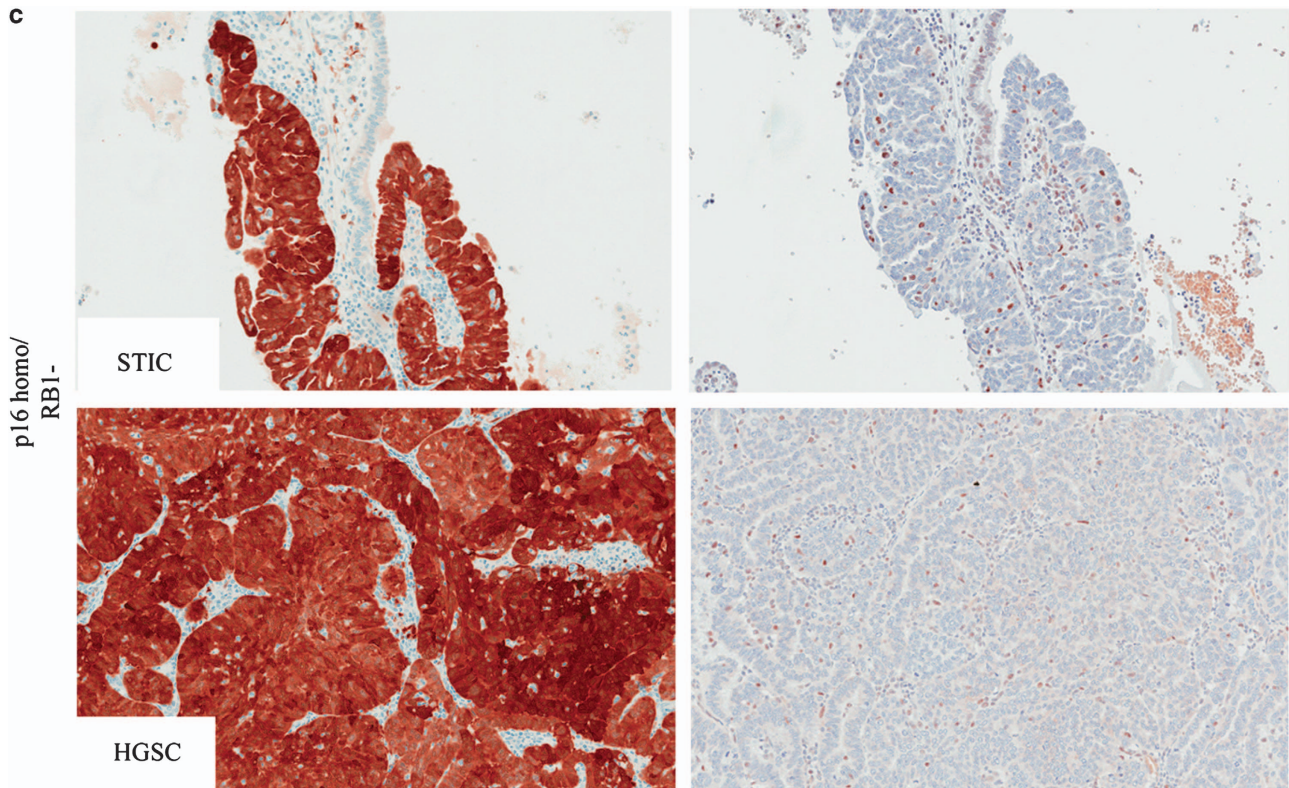


Figure 4 Continued.

Although, we were not able to identify any morphological differences among the 3 p16/Rb subgroups, in the cohort of 75 profiled tumors, a thorough investigation of morphological features on the entire set of 334 high-grade serous tumors is warranted.

We have also demonstrated that these clinically distinct subgroups are present in serous tubal intraepithelial carcinomas, the commonly accepted high-grade serous carcinoma precursor lesions, indicating that retinoblastoma pathway deregulation is an early event and that tumor behavior may be established early in ovarian tumorigenesis.

The striking p16 homogeneous expression pattern, frequently used as a marker of RB1 loss, seen in approximately 66% of high-grade serous carcinoma and uncommon in other epithelial ovarian cancer histotypes,^{7,25–28} predicts for a decreased progression-free survival and overall survival. The heterogeneous p16 pattern, present in about 33% of high-grade serous carcinoma cases and frequently seen in other epithelial ovarian cancer, predicts a better clinical outcome for high-grade serous carcinoma patients. Interestingly, loss of the *CDKN2A* gene is uncommon in high-grade serous carcinoma, found in only 6% (7/75) of our tumors. Copy number deletions in *CDKN2A* were infrequent and only three out of seven of those identified correlated with loss of protein expression, suggesting that retinoblastoma pathway dysfunction by *CDKN2A* loss occurs, but is an infrequent event in high-grade serous carcinoma.

The most common alteration associated with p16 homogeneous expression is loss of *RB1*. However, alterations in *RB1* alone did not predict for clinical outcome. The observed loss of RB1 protein in approximately 30% of samples, correlated strongly with *RB1* allelic loss and *RB1* gene alterations (surveillance of deletions and mutations). *RB1* inactivation through large spanning gene deletion occurred at a higher frequency (38%) than previously reported.^{5,29} This is an interesting observation given the location of the *RB1* gene on chromosome 13q14.2, a chromosomal arm not commonly reported as a deletion hotspot in high-grade serous carcinoma.⁵ Our mutation analysis and sequencing data are consistent with that of the TCGA research network results, showing infrequent *RB1* point mutations in high-grade serous carcinoma.^{5,30}

Although high p16 is frequently used as a marker of *RB1* loss, this is not the case in approximately 30% of our high-grade serous carcinoma samples, which show the shortest progression-free survival and shortest overall survival. The mechanism of p16 overexpression in the presence of functional RB1 is uncertain. Human papillomavirus infection, known to inactivate RB1 through binding to E7 proteins, was excluded by human papillomavirus testing (data not shown). There are few *RB1* mutations known to maintain expression of the protein and few identified *RB1* point mutations in high-grade serous carcinoma. Alternatively, alterations in *CCNE1* may result in a bypass of the retinoblastoma

pathway in this context, triggering p16 overexpression to inhibit continued proliferation.¹² *CCNE1* amplification is a known predictor of poor clinical outcome,³¹ but does not account for all of the cases in the p16 homo/RB1+ subgroup. Other yet unidentified oncogenes may also contribute to the retinoblastoma pathway deregulation in this particular subgroup.

Mechanisms of retinoblastoma pathway deregulation in other tumor types have previously been associated with clinical parameter differences including type, recurrence, and outcome,^{7,8,25,26,32,33} and can be exploited for targeted therapy approaches to identify the most responsive patient subgroups.^{33–35} However, our study is unique because we have identified differential clinical behavior in a specific cancer histotype. This knowledge may have implications for identifying specific treatment strategies to improve care. Patients in the p16homo/RB1– subgroup show a significant increase in overall survival (>60 month; $P=0.013$), which suggests an increase in sensitivity to cytotoxic agents. In addition, this stratification could be used to determine novel second-line treatment options such as CDK4 inhibition (which inhibits the interaction with cyclin D1 resulting in hypophosphorylation of RB1 and E2F inactivation).¹⁵ Konecny *et al.* reported that ovarian cancer cell lines with *RB1* deletions and *CCNE1* gains are more resistant to CDK4 inhibitors, whereas those with *CDKN2A* gene deletions and decreased protein expression were more sensitive.¹⁵ We found that *CDKN2A* and/or *CDK4* gene deletions are rare in high-grade serous carcinoma and that cyclin D1 overexpression is predominant in the p16 hetero/RB1+ tumors. Patients within the p16 hetero/RB1+ subgroup may be predicted to benefit most from CDK4 inhibitor treatments, in contrast to patients with cyclin E1 gains/amplifications in the p16 homo/RB1+ subgroup and *RB1* deletions in the p16 homo/RB1– subgroup. Similarly, some high-grade serous carcinoma patients may optimally benefit from topoisomerase II inhibitors such as etoposide, which causes double-stranded DNA breaks and cell death by trapping topoisomerase proteins to DNA.^{36–38} Lung carcinoma studies indicate that *RB1* mutations (found in 90% of small cell lung tumors) confer sensitivity to this treatment,^{9,35,39} suggesting that patients within the p16 homo/RB1– subgroup may also benefit from etoposide.

In summary, we have demonstrated by comprehensively assessing retinoblastoma pathway members: that epithelial ovarian cancer classified as high-grade serous carcinoma have non-synonymous mechanisms of retinoblastoma pathway deregulation, that these mechanisms result in different patterns of p16/RB1 protein expression, which stratify most high-grade serous carcinoma into three clinically relevant sub-groups, and that these alterations occur early in tumor development.

Further studies in clinical trial settings would validate the utility of the retinoblastoma pathway subgroups in predicting the efficacy of novel targeted therapies.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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BRCA and early events in the development of serous ovarian cancer

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Women who have an inherited mutation in the BRCA1 or BRCA2 genes have a substantial increased lifetime risk of developing epithelial ovarian cancer (EOC), and epidemiological factors related to parity, ovulation, and hormone regulation have a dramatic effect on the risk in both BRCA mutation carriers and non-carriers. The most common and most aggressive histotype of EOC, high-grade serous carcinoma (HGSC), is also the histotype associated with germline BRCA mutations. In recent years, evidence has emerged indicating that the likely tissue of origin of HGSC is the fallopian tube. We have reviewed, what is known about the fallopian tube in BRCA mutation carriers at both the transcriptional and translational aspect of their biology. We propose that changes of the transcriptome in BRCA heterozygotes reflect an altered response to the ovulatory stresses from the microenvironment, which may include the post-ovulation inflammatory response and altered reproductive hormone physiology.

Keywords: BRCA, fallopian tube epithelium, high-grade serous carcinoma

INTRODUCTION

In 2013, about 22,240 women in the United States would have been diagnosed with invasive epithelial ovarian cancer (EOC) and an estimated 14,000 women with EOC would have died (1). There are five major histotypes of EOC and they are distinct epidemiologically, phenotypically, and molecularly, namely: mucinous, endometrioid, clear cell, low-grade serous, and high-grade serous carcinoma (HGSC). Of these, HGSC is the most prevalent histotype in the Western Hemisphere, the most lethal, typically is diagnosed at an advanced stage, and there are no effective cancer screening strategies. More than 75% of women with this diagnosis will succumb to the disease after combined first line treatment, which includes surgery and adjuvant platinum-based chemotherapy, with a 5-year survival of <30% (1, 2). HGSC is a genetically unstable tumor, characterized by a varied histomorphology unified by marked pleomorphism, a high mitotic rate, and biomarker expression reflective of the most common molecular alterations. The latter includes the near ubiquitous presence of a mutation in the tumor suppressor p53 (TP53), resulting in either over accumulation of p53 protein by immunohistochemistry (missense – 60% of analyzed cases) or complete loss of protein expression (frameshift/splicing junctions/non-sense – 39% of analyzed cases) (3). Mutations of p53 are present in early stage HGSC, and mutant TP53 is likely an essential driver mutation required for the early pathogenesis of HGSC (4). Other recurrent mutations in HGSC are infrequent, but most prominently include BRCA1 and BRCA2, with BRCA germline mutations seen in 13–16% (5), and somatic mutations seen in about 6% of cases.

High-grade serous carcinoma is the predominant histotype associated with hereditary breast-ovarian cancer (6, 7). Women with inherited mutations of BRCA1 or BRCA2, have a lifetime risk of 40–60% (BRCA1) and 11–27% (BRCA2) (8–12).

Women known to be at increased genetic risk based on family history and/or genetic testing are offered risk-reducing salpingo-oophorectomy (RRSO), which reduces the risk of malignancy by up to 96% (13, 14) and is usually performed after completion of childbearing and while the woman is still pre-menopausal (13, 15). An unexpected finding on histopathology review of the resected fallopian tubes in this population was the presence of clinically undetected, occult carcinomas in the fallopian tubes, a tissue previously thought to develop carcinomas only rarely. These were seen more frequently than in the ovarian tissues (16). This discovery was followed by careful review of the fallopian tube tissues, and subsequent studies have reported histological lesions purported to be HGSC precursors in the fallopian tube epithelium – these had not been found in the genetic high-risk ovarian tissues (16–22). Hence, detailed histo-pathological examination of the resected ovaries and fallopian tubes in BRCA mutation carriers has led to a radical change in existing paradigms of serous carcinogenesis. Because loss of BRCA function is frequent in HGSC, study of the effect of BRCA, including heterozygosity/haploinsufficiency and loss of function in the fallopian tube epithelium prior to the development of HGSC, offers opportunities to better understand HGSC pathogenesis, and should lead to the development of novel and more effective preventative, and possibly, screening strategies.

BRCA1 AND BRCA2 AND HIGH-GRADE SEROUS CANCER

Molecularly, the breast cancer susceptibility genes (BRCA) BRCA1 and BRCA2 can sense DNA damage and are involved in DNA repair via interactions with RAD51 (23–25); these three proteins are essential for genomic stability in normal cells predominantly through the homologous recombination pathway (HR) (26). BRCA1 is a known modulator of the cell-cycle at the G2-M checkpoint (27) operating through co-activation with p53 (28)

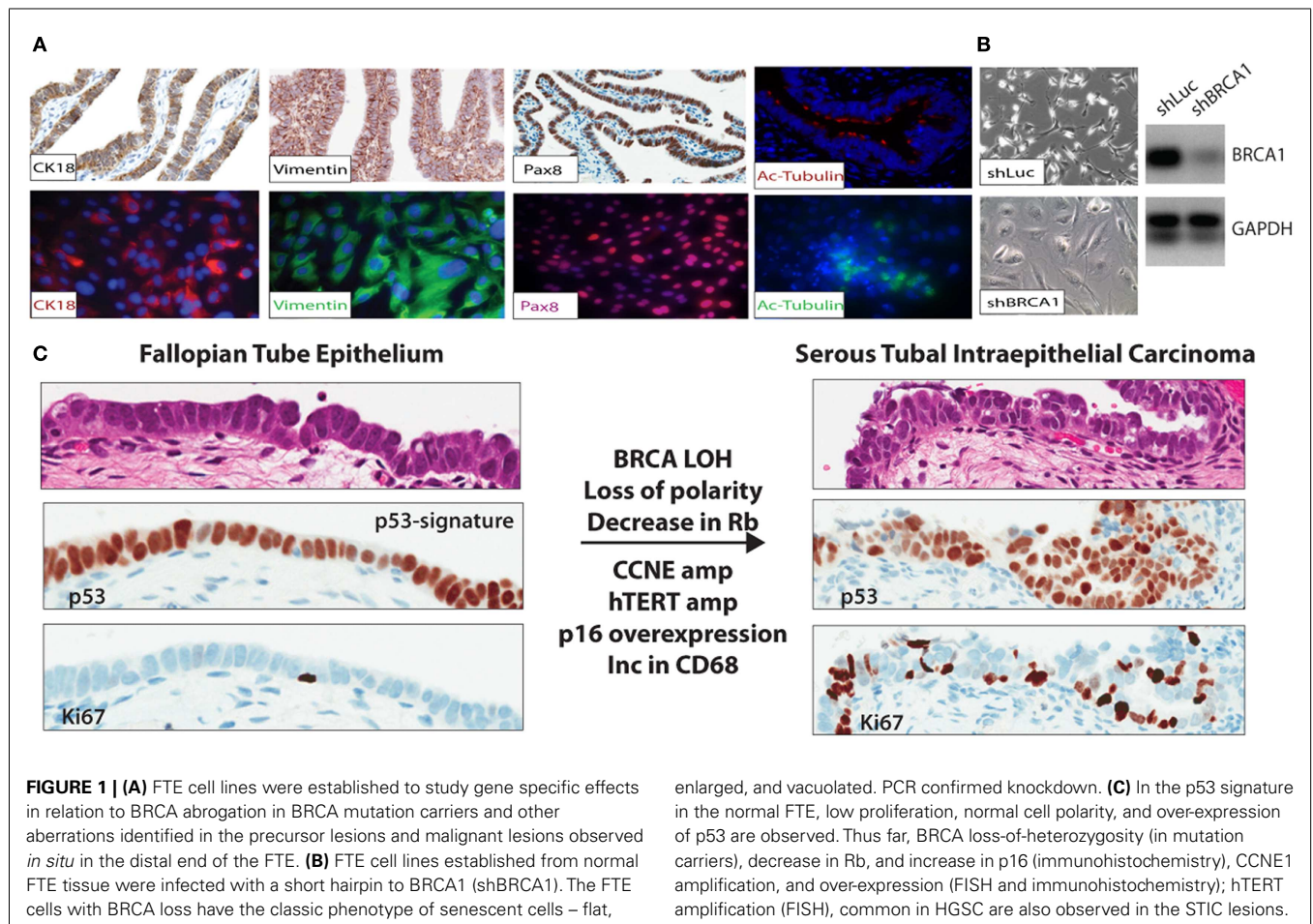
and has also been shown to epigenetically regulate the oncogenic microRNA 155 and to maintain heterochromatin structure via ubiquitylation of H2A (29, 30). Inherited mutations in BRCA1/BRCA2 confer an autosomal-dominant effect and range from being deleterious to protein function to being of uncertain significance (31).

Breast cancer susceptibility gene mutation carriers develop cancers in hormonally regulated tissues, most frequently in breast and ovarian/tubal tissues, but a unifying mechanism of early malignant transformation in these tissues is not known. The BRCA associated carcinomas share some common features including a high-grade phenotype, frequent mutations of TP53, and other copy number landscape features like Cyclin-E amplification and deletion of Rb (32). Altered BRCA function in HGSC does not only occur in the setting of hereditary disease. Dysfunction of BRCA1 or BRCA2 is prevalent in patients with HGSC via 6% somatic mutations (5, 33–35); 13–31% promoter hypermethylation (5, 36–38); 7.9–17% amplification of EMSY (5, 39, 40); or 13.2% promoter hypermethylation of FANCF (41). The sum of these genomic alterations predominantly in the HR pathway of HGSC has led to determining the “BRCAness” profile in patients (42, 43). BRCAness is defined as a phenotype determined by deficiencies in the double strand break (DSB) repair pathways, as seen in tumors associated with germline BRCA mutations and a subset of sporadic high-grade serous ovarian cancers. An understanding of the

early molecular changes in genetic high-risk patients may therefore also be of importance to many of the sporadic cancers. Patients with the BRCAness profile most likely will benefit from treatments affecting other DNA repair pathways – specifically PARP inhibitors (43). Outcome data suggests that patients with loss of function of BRCA have improved survival, but recently a study by McLaughlin and colleagues determined that although BRCA mutation carriers have a short-term (up to 5 years post diagnosis) benefit and response to platinum-based therapy, there is a lack of long-term (up to 10 years post diagnosis) survival benefit (44). Most promisingly, the loss of function of BRCA1/BRCA2 whether genetic or epigenetic by mechanisms including promoter hypermethylation, offers the possibility of improved therapies with poly (ADP-ribose) polymerase (PARP) inhibitors (43).

BRCA1 AND BRCA2 AND THE FALLOPIAN TUBE EPITHELIUM

The mechanisms underlying malignant transformation in these estrogen responsive tissues are poorly understood, but likely involve loss of heterozygosity of the remaining wild type BRCA allele (45) in addition to inactivation of p53. During ovulation, it is thought that high levels of reactive oxygen species (ROS) are released via the cytokine surge accompanied with lysis of the ovum (follicular fluid). These species have a complex role in the development and progression of cancer (46). The high ROS levels are likely a source of “carcinogens,” which cause DNA damage in the



FTE and possibly contribute to the mutations in TP53. In normal cells repair of DNA damage results in cell-cycle arrest through senescence or death as demonstrated in epithelial cell lines established from FTE (**Figures 1A,B**). This process must be overcome for transformation to occur (47). In high-grade serous ovarian cancer cells, 99% of tumors have a mutation in TP53, indicating that the mutation likely occurs early in disease progression (3, 5). This combination – TP53 mutation and BRCA loss, can provide an escape or by-pass through the cell-cycle checkpoints to allow additional cancer promoting mutations, amplifications, or deletions. Therefore, BRCA1/BRCA2 deficient cells [lacking ATM/ATR-CHK2 pathway (48)] cannot sense DNA damage in order to transduce signal to the already TP53 mutant cells. In this setting, cells can overcome the barriers for cell-cycle progression, however this may not be sufficient for transformation into a tumor.

In normal cells of mutation carriers, only one allele is mutated, and BRCA1 function is presumed to be intact. This may however not be true, as evidence in support of BRCA1 haploinsufficiency accumulates. For example, in normal human mammary epithelial cells from BRCA1 heterozygotes, DNA homologous repair is suppressed (49). BRCA1 haploinsufficiency may be an early but not a sufficient step of BRCA1-mediated breast carcinogenesis. In HGSC, it is uncertain when during malignant transformation of FTE, loss of BRCA1 function occurs. In contrast to breast cancer, it seems likely altered p53 function resulting from p53 mutation occurs prior to loss of the wild type BRCA1 allele in FTE transformation. Loss of BRCA1 protein and loss of heterozygosity is seen once malignant transformation has occurred but, according to Norquist et al. not in early precancerous lesions (45). The p53 mutation is thought to promote genomic instability, a hallmark of high-grade serous cancer, and cooperates with BRCA1 loss or a dysfunctional HR pathway to mediate the extent of genomic amplifications and gains so commonly seen in HGSC.

p53 SIGNATURE AND SEROUS TUBAL INTRAEPITHELIAL CARCINOMA

For many years, in the absence of a reproducible histological precursor lesion of HGSC, the cell of origin was presumed to be the ovarian surface epithelium (OSE), a modified type of mesothelium. Detailed histo-pathological examination of tubal epithelia (FTE) in the genetically high-risk population undergoing risk-reducing surgery has led to the discovery of putative cancer precursor lesions in the fallopian tube, some of which, i.e., the p53 signature – described as a string of 10–12 histologically normal secretory (non-ciliated) cells expressing the TP53 protein with a low proliferation rate (Ki67) (50), are found with a similar frequency in BRCA mutation carriers and non-carriers. Two independent studies reported similar findings albeit at different frequencies of p53-signatures between the two study cohorts: 11 and 19% (51) and 24 and 33% (52) in women with germline BRCA mutations and population control, respectively. The cells within the p53 signature are Pax8 positive and up-regulate phosphorylated – γ H2AX, reflective of concomitant DNA damage. Women with an inherited mutation in the TP53 gene – the Li Fraumeni syndrome, have an increased risk in developing between five and six different cancers (breast, brain, soft tissue sarcomas, and blood cancers) throughout their lifetime (52). These patients, however, do not have an increased incidence of developing high-grade

serous ovarian cancer, but have an increased number of p53-signatures compared to the rest of the population. In addition, in a small epidemiological study, p53-signatures were not associated with the traditional risk factors of breast-feeding, parity and tubal ligation, bringing into questions whether the p53 signature is a true cancer precursor lesion (53). However, it can be said that loss of normal p53 function is necessary, but not sufficient to promote carcinogenesis of epithelial cells in the distal fallopian tube.

Occult invasive carcinoma and serous tubal intraepithelial carcinomas (STICs) were identified in the fallopian tubes of mutation carriers undergoing risk-reducing surgery, with an incidence of about 4–6% for occult cancers (16, 54, 55). Importantly, STICs are found not only in BRCA mutation carriers, but are also detected in about 60% of sporadic HGSC (19, 56). STICs are thought to have progressed from the p53 signature and are characterized as being highly proliferative (>10% Ki67) (57), show loss of apical to basal nuclear polarity and, in common with HGSC, demonstrate: over-expression of cyclin-E (58), amplification of hTERT (59), p16 over-expression (CDKN2A), loss of Retinoblastoma protein (Rb) (60), and up-regulation of the PI3K pathway (61) (**Figure 1C**). In mutation carriers undergoing RRSO, STICs were identified in at least 8% of cases, a higher frequency than seen in patients at low genetic risk (51, 52, 62, 63).

Like HGSC, the frequency of STIC lesions increases with age, is increased in BRCA1/2 mutation carriers, and is lower with oral contraceptive use, all features providing further evidence that STIC is an immediate precursor of invasive and clinically detectable carcinoma (53). These intraepithelial carcinomas should not be considered as only *in situ* carcinomas, because in at least some cases while tumor cells do not invade underlying stroma, they can detach, and because of the accessibility of the ipsilateral ovary and other peritoneal surfaces to the tubal fimbria, cells may implant and establish tumor growth in other sites. Currently, little evidence exists that patients with only a diagnosis of STIC require adjuvant therapy (64). Further molecular and genetic characterization of STIC is ongoing, but molecular evidence to date indicates that alterations commonly seen in HGSC are also present in STIC. Lesions that precede the STIC, are not well characterized, but currently the term serous tubal intraepithelial lesion (STIL) is given to lesions according to criteria recommended in a proposed diagnostic algorithm. The STIL is described as a lesion, which has abnormal p53 expression by immunohistochemistry and increased proliferation relative to background (tubal epithelium) but <10% Ki67 positive. (57, 65). Ongoing studies are required to further define this lesion as current definitions lack diagnostic reproducibility. Other than the changes associated with the p53 signature, molecular changes which precede the establishment of an intraepithelial cancer are not well documented. Indeed, these lesions are uncommon, and identified with poor reproducibility.

NORMAL TUBE EPITHELIUM IN BRCA MUTATION CARRIERS

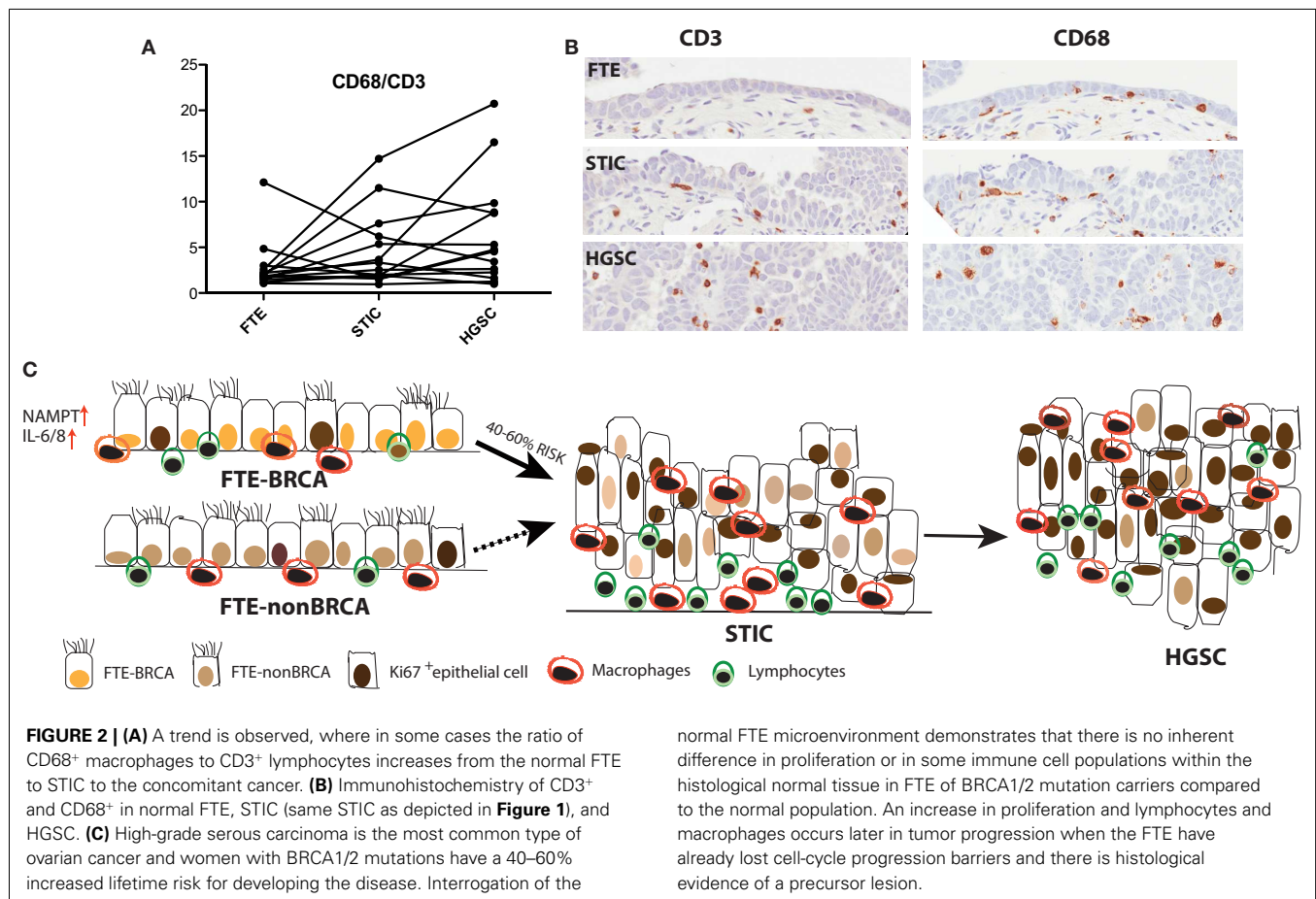
Hormonally responsive epithelia, from breast, ovary, and fallopian tube, are the preferential targets for malignant transformation in BRCA1/2 mutation carriers and the mechanisms are increasingly being determined primarily through studying breast epithelia (66). Evidence is emerging nonetheless that morphologically normal

fallopian tube epithelium from women with inherited mutations, differs significantly from the tubal epithelium of women at low cancer risk. Differences in morphologically normal epithelium from BRCA mutation carriers have shed light into the effects of heterozygosity and predisposition to high-grade serous ovarian cancer. In five previous reports, we have used morphologically normal fallopian tubal epithelium from BRCA1 and BRCA 2 (FTE-BRCA) mutation carriers and non-mutation carriers (FTE-normal), to compare gene expression profiles to identify differences conferred by the presence of one mutant allele (67–71). In addition to family history, a major risk factor is number of lifetime ovulations, and oral contraception use and increase in parity lead to a reduction in EOC risk (72). The formerly prevailing incessant ovulation hypothesis first described by Fathalla suggested that continuous disruption and surrounding inflammation of the OSE during ovulation led to the development of carcinoma in the ovary (73). It is likely however that the effects of ovulation are still important in malignant transformation, but the effects are on fimbrial, not ovarian, epithelium.

Therefore in the design of experiments, the patient tissues analyzed were controlled for not only age and menopause but also stage in the ovarian cycle – follicular (proliferative phase) and luteal (post-ovulatory phase) at the time of surgery (70, 71). We showed that the BRCA mutation in morphologically normal fallopian tube epithelium confers a significantly altered gene expression signature. Some of these altered pathways include

the TGF- β pathway, MAP kinase pathway, the adipokine signaling pathway, inflammatory pathway, and the p53-signaling pathway (70, 71). In particular genes involved in DNA damage and inflammation were validated as both having transcriptional and translational differential expression in the normal fallopian tubes (ampulla and fimbria) of BRCA mutation carriers. Namely, DAB, NAMPT, C/EBP- δ , GADD45 β , and NF- κ B are genes involved in the Jak/Stat, DNA damage, and TGF- β pathways and are prominently differentially expressed in mutation carriers and in HGSC. In these studies, we noted, that BRCA mRNA levels were not substantially different between carriers and non-carriers, indicating that the wild type allele was still intact. In an independent study, Press et al. reported significant differences in proliferation and cell-cycle regulation in BRCA mutation carriers (with and without occult carcinoma) (74).

We subsequently analyzed the distal end FTE (the fimbria), the anatomical region of highest risk and the ampulla for: (1) the presence of immune infiltrates (CD3⁺ and CD8⁺ lymphocytes and CD68⁺ macrophages) and (2) the proliferation status of FTE cells in both BRCA mutation carriers and population control. This study although not exhaustive, revealed that independent of BRCA mutation status: (1) macrophages were more prevalent in the luteal phase than the follicular phase of the ovarian cycle and (2) proliferation in FTE cells is predominantly an effect of the follicular phase rather than BRCA mutation status in histologically normal tissue (**Figure 2**). However, a small subset of FTEs



from BRCA2 mutation carriers had a diffuse increase in proliferation in the absence of histological lesions, but overall there was no statistical difference in proliferation compared to the control tissues (68). Therefore, we propose that chronic inflammatory states through cyclical ovulation and the presence of a mutated BRCA allele can predispose the normal FTE to develop lesions, which may lead to serous carcinoma. We hypothesize that this occurs through deregulation of DNA damage response genes and synergistically through up-regulation of cytokines, pro-inflammatory, and proliferation genes. It is possible that changes demonstrated in gene expression profiles reflect the earliest alterations in cancer development, and/or that they are markers of increased cancer risk.

OVULATORY CYCLE AND BRCA IN THE FALLOPIAN TUBE EPITHELIUM

Most women who develop sporadic cases of EOC are peri- or postmenopausal with a mean age of 58 years (75); however, BRCA1 mutation carriers develop the disease earlier with a mean age of 51 years and BRCA2 mutation carriers a bit later, with a mean age of 57 years (75–78). In addition to family history, the major epidemiological risk factors for EOC indicate a strong influence of reproductive factors and reproductive hormones. Risk factors including nulliparity, early age of menarche, late age of menopause, hormone replacement, obesity, and protective factors including oral contraceptive use, indicate an association with increased lifetime ovulations and/or greater lifetime exposure to estrogen. A higher risk of ovarian cancer has been reported with cyclical use of hormone replacement therapy rather than continuous use or any use of estrogen or progestin after menopause (79) for both BRCA mutation carriers and non-carriers (72).

The influence of sex hormones on tubal/ovarian malignant transformation is not well understood, but seems likely that the BRCA1/2 associated changes in reproductive hormones and their receptors play a role in tumor formation, in addition to the alterations in DNA damage repair. BRCA1/2 mutation carriers do not have menopause at an early age (80). Higher circulating estradiol is associated in the general population with a pre-menopausal breast cancer risk, and BRCA2 carriers with breast cancer do have higher estradiol levels in the early follicular phase, but a similar association with circulating progesterone is not seen. It has recently been shown that mutation carriers have higher levels of both estradiol and progesterone during the luteal, not follicular phase, leading the investigators to suggest a defect in steroid hormone regulation potentiates the mutagenic effect of the BRCA mutation (80, 81). In mice, it has been shown that granulosa cells in mice lacking functional *Brca1* are exposed to increased estradiol stimulation due to a combination of a prolonged pre-ovulatory (proestrus) phase of the estrus cycle and increased levels of circulating estradiol. In addition, estrogen biosynthesis in granulosa cells is altered in mice not only with a deleterious homozygous mutation but also in mice with a heterozygous *Brca1* mutation (82), a state which mimics the BRCA1 mutation carriers. This provides further evidence that heterozygous BRCA1 mutations are associated with phenotypic changes.

The role of estrogen and progesterone in early malignant transformation in the FTE is not yet well understood. Estrogen mediates its action primarily through the estrogen receptor (ER α and ER β).

Estrogen stimulates the expression of a number of genes that promote cell proliferation, motility/invasion, and inhibition of apoptosis namely: IL6, TGF- α , EGF, PI3K/Akt, IGF-1, and Bcl-2 (which is predominantly expressed in secretory FTE) (78). The estrogen dominant phase during the ovarian cycle is the follicular (or proliferative) phase and is associated with an increase in FTE proliferation (68) and promotion of ciliogenesis (83). In contrast, progesterone receptor activity is associated with a decrease in cell proliferation (68), an increase in apoptosis, possibly mediated through the down-regulation of CDK1/cyclin B1 complex, which impedes the G2/M transition. Conversely, in the breast, it is known that progesterone elicits proliferation through Cyclin D1 in PR positive cells (a cell intrinsic autocrine loop) and in PR negative cells via NF- κ B ligand RANKL secretion (paracrine) (84, 85). Progesterone mediates its activity through the progesterone receptors (PR-A and PR-B are isoforms with differential translational start sites). On progesterone binding PR translocates to the nucleus to direct an antagonist effect on ER α signaling. Both ciliated and secretory FTE cells express the estrogen and progesterone receptors (69) and undergo cyclic changes in growth and differentiation throughout the ovarian cycle; these changes are most evident in the fimbriae (86) the “high-risk” zone of the tube (86). The fallopian tube epithelia in the luteal phase of the ovarian cycle have significantly lower levels of the progesterone (PR-A) (69) and estrogen [ER α] receptors (87)] (Figure 3).

During ovulation, there is a surge of estrogen released into the FTE microenvironment with release of follicular fluid, which contains high estrogen levels. This effect might be exacerbated (88) in fallopian tube epithelia of BRCA mutation carriers under the direct influence of the relevant DNA repair pathways, which are potentially dysfunctional. In addition to its well-established roles in regulation of DNA damage response, the *Brca1* protein inhibits ER α transcriptional activity through direct action of BRCA1 and ER α proteins and down-regulation of p300, a nuclear receptor co-activator (89). *Brca1* protein also regulates estrogen receptor action through suppression of aromatase, the enzyme required for estrogen biosynthesis from androgen. Gorrini et al. recently showed that an antioxidant estrogen target gene – Nrf2, can mediate a pro-survival effect in the absence of normal BRCA1 protein, in which cells would otherwise undergo cellular senescence or death (66). BRCA1 loss in mammary epithelium therefore alters the estrogenic growth response, and increased estrogen signaling collaborates with *Brca1* deficiency to accelerate preneoplasia and cancer development. Although this has not been tested in FTE, this is an interesting concept that may have implications in serous carcinogenesis.

A decrease in the transcription and translation (by immunohistochemistry) of PR-A and PR-B were observed in the luteal phase of both BRCA mutation carriers and population control (69). PR gene signatures were identified in a subset of FTE cases in the luteal phase that had a similar profile to HGSC, however, PR target genes were not differentially expressed between BRCA mutation carriers and controls (69). In HGSC, PR expression is predominantly decreased/lost, a finding in 70–80% of patients (69, 90). PR expression in greater than 50% of tumor cells has been recently reported to have an overall survival benefit, and this benefit was independent of germline BRCA1/2 mutation status

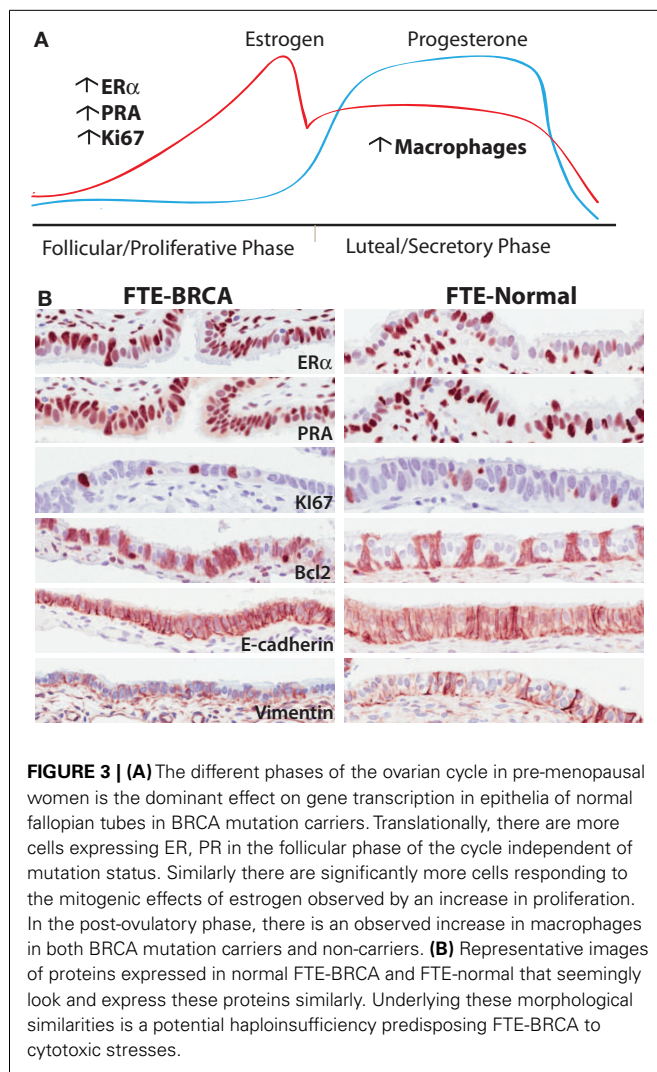


FIGURE 3 | (A) The different phases of the ovarian cycle in pre-menopausal women is the dominant effect on gene transcription in epithelia of normal fallopian tubes in BRCA mutation carriers. Translationally, there are more cells expressing ER, PR in the follicular phase of the cycle independent of mutation status. Similarly there are significantly more cells responding to the mitogenic effects of estrogen observed by an increase in proliferation. In the post-ovulatory phase, there is an observed increase in macrophages in both BRCA mutation carriers and non-carriers. **(B)** Representative images of proteins expressed in normal FTE-BRCA and FTE-normal that seemingly look and express these proteins similarly. Underlying these morphological similarities is a potential haploinsufficiency predisposing FTE-BRCA to cytotoxic stresses.

(90). In contrast, 70–80% of HGSC patients express ERα (>50%) but ER expression has not been shown to be associated with a significant recurrence free progression or survival benefit (87, 90).

Epidemiological data indicate that HGSC risk is closely linked to the events of ovulation, and these risk factors and protective factors for the most part are true for both sporadic and hereditary HGSC. In addition, evidence suggests that the risk for EOC increases during the pre-menopausal years, and that menopause is protective against ovarian cancer (91). The role of sex hormones in ovarian cancer development is complex however, and early evidence suggests that endocrine function may differ in BRCA1 heterozygotes. The mechanisms of altered hormone function and impact of genetic mutations on endocrine production and receptivity in the FTE of high-risk patients is not yet understood, but it remains possible that the underlying growth stimulatory effects of estrogen are altered in a BRCA mutation carrier.

CONCLUDING REMARKS

In conclusion, there are many epidemiological studies linking ovulation, parity, and hormonal use to the development of EOC.

About 60% or more of HGSC demonstrate a BRCAness profile predominantly through a dysfunctional homologous recombinant pathway, which synergizes with the ubiquitousness of the p53 mutations found amongst these tumors. In the normal fallopian tube of BRCA mutation carriers, transcription profiles reveal predominant differences in DNA damage and inflammation pathways. Interestingly and may be not surprisingly, FTE-BRCA samples are transcriptionally indistinguishable from FTE-normal samples when transcription profiles undergo unsupervised hierarchical clustering (70). Instead, the sample alignment is dependent on the estrogen or progesterone dominant phases of the ovulatory cycles, lending biological support to the known epidemiological risk factors and providing evidence for a possible haploinsufficiency of the functional allele in the normal FTE.

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Figure 1

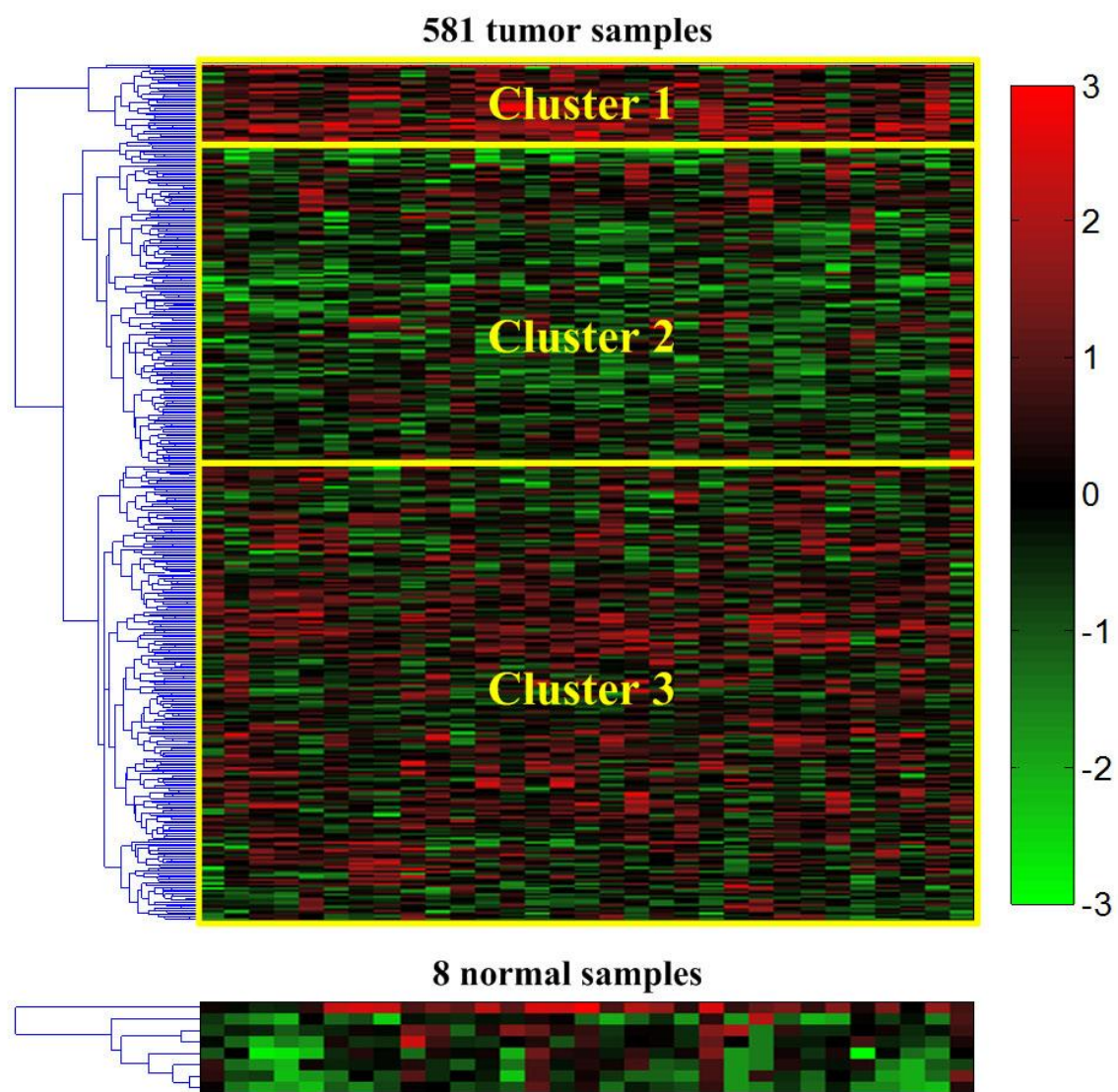


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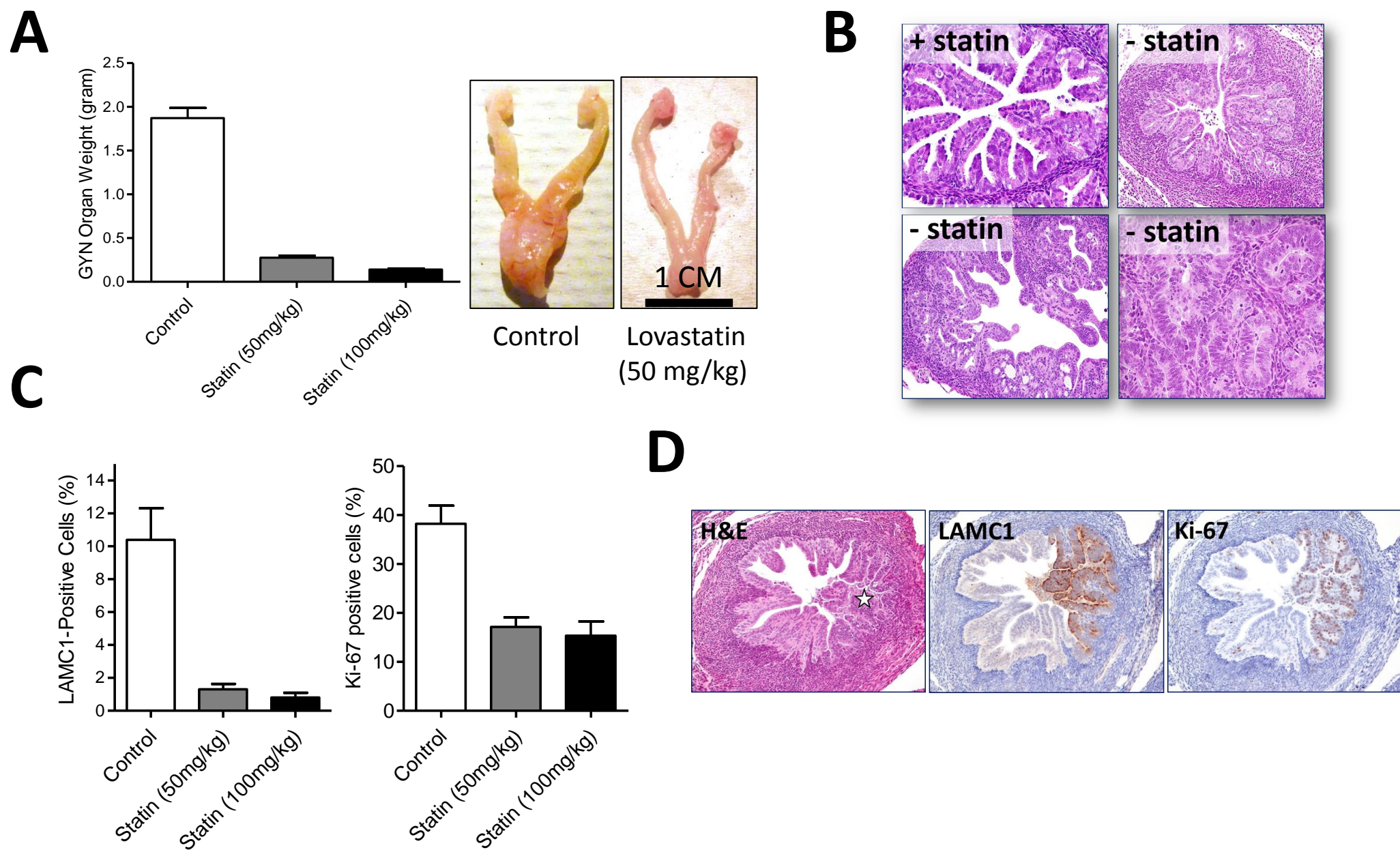
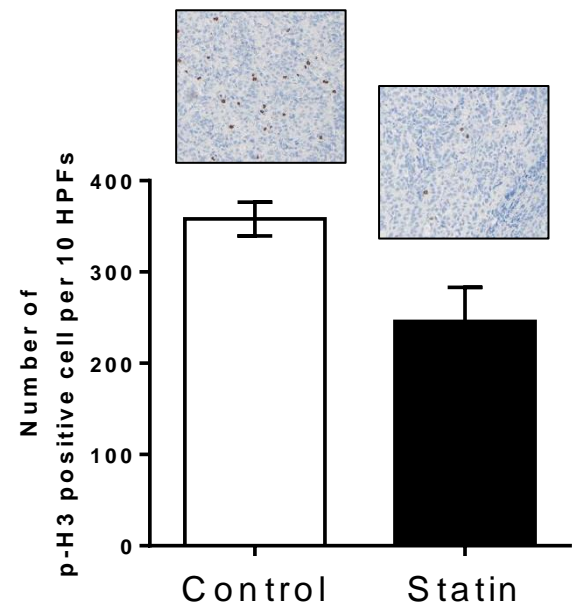
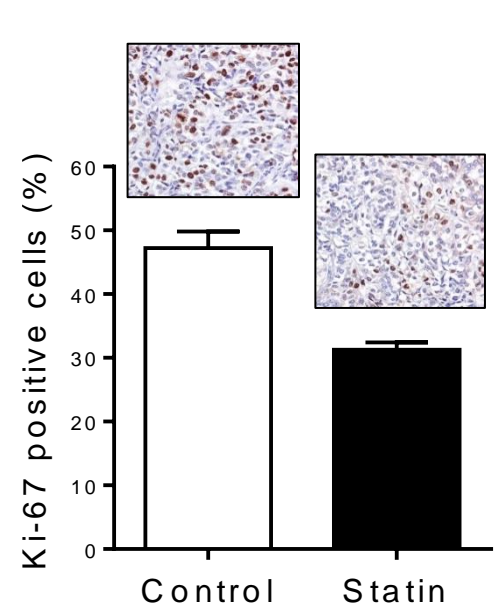
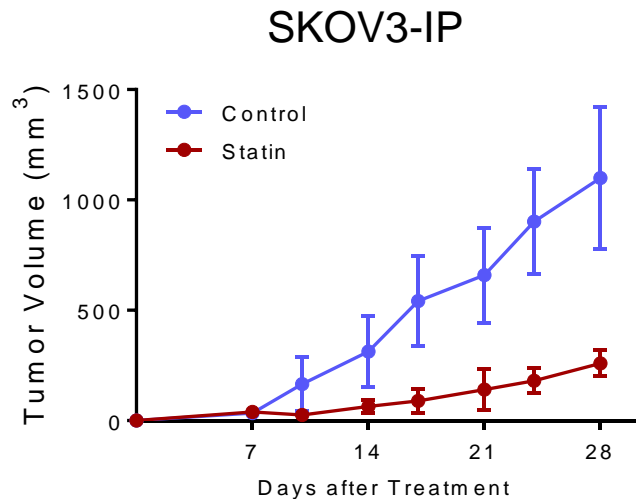


Figure 3

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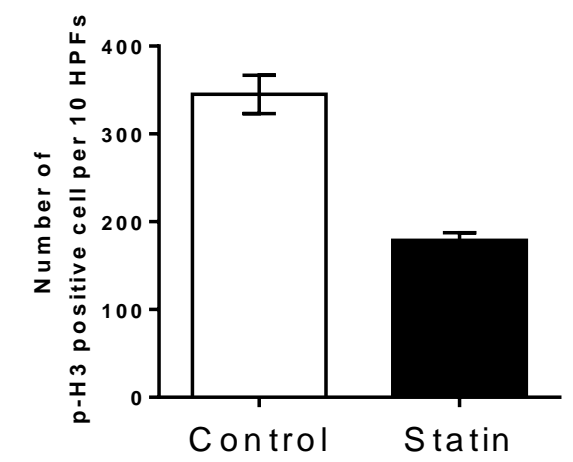
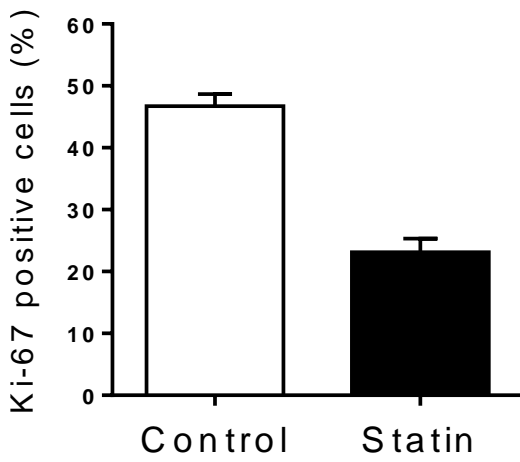
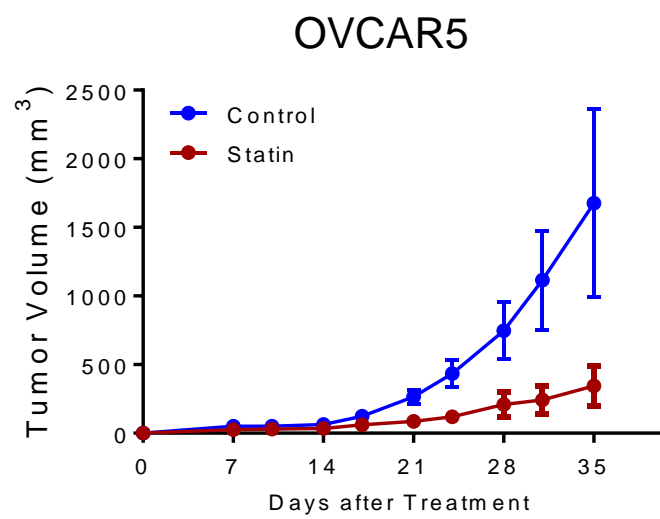


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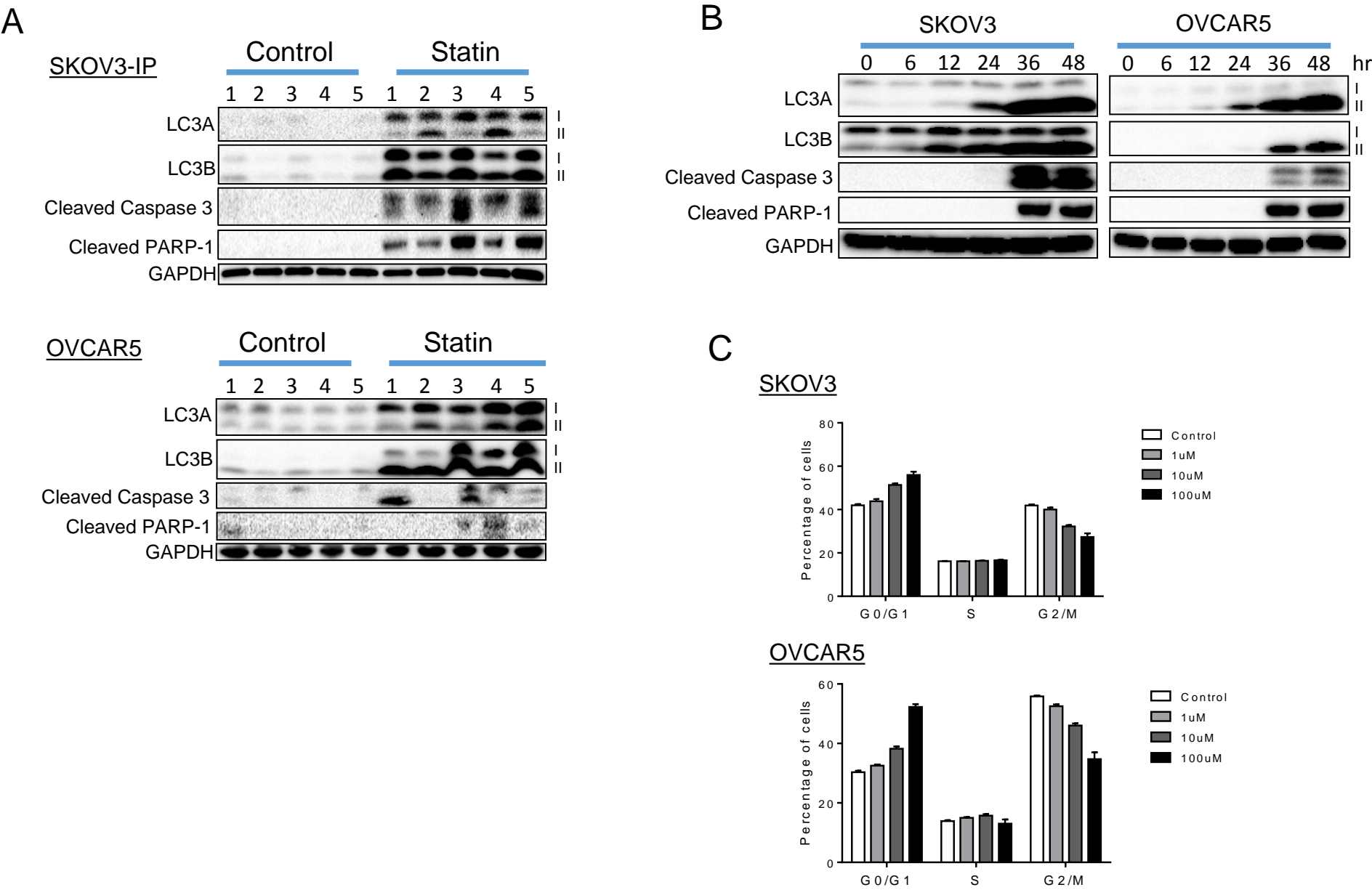
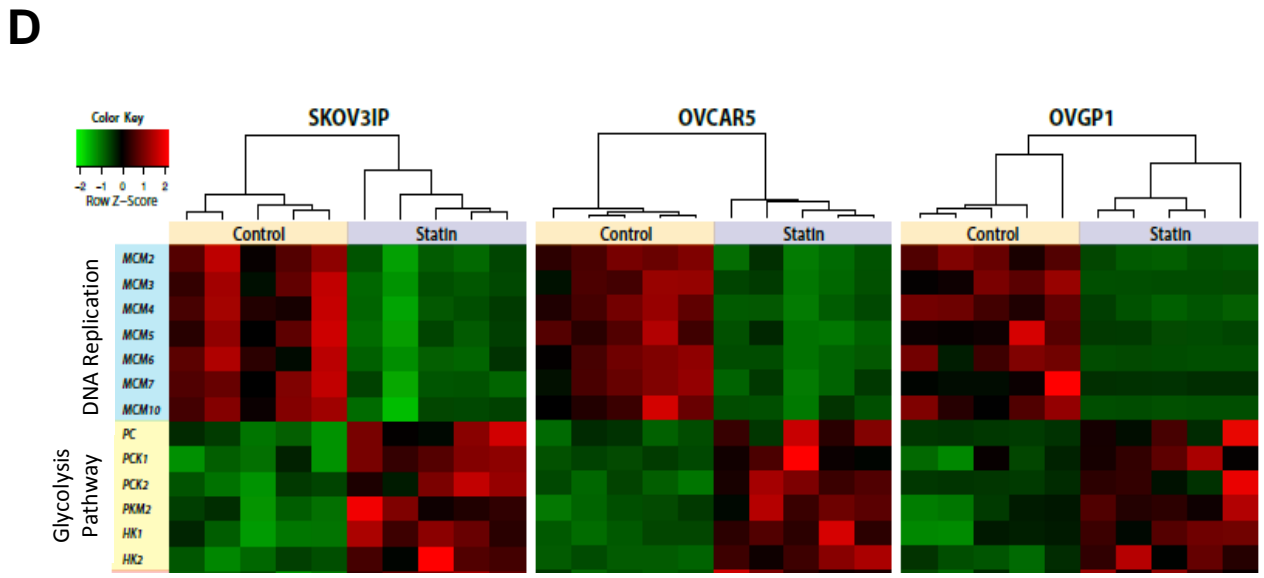
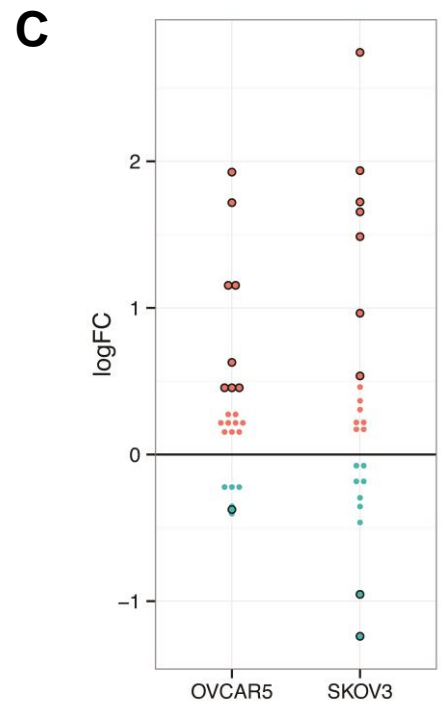
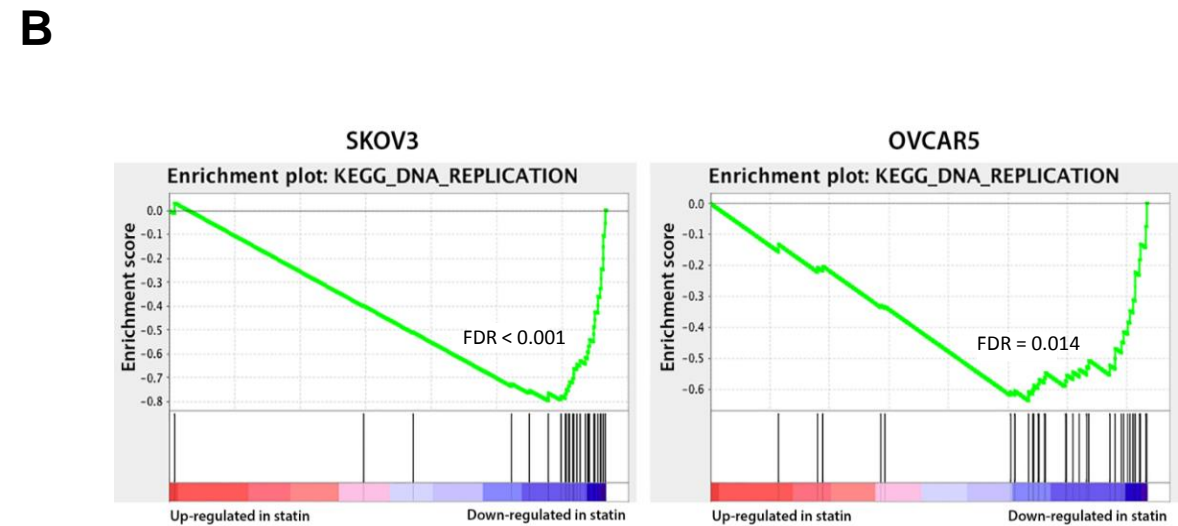
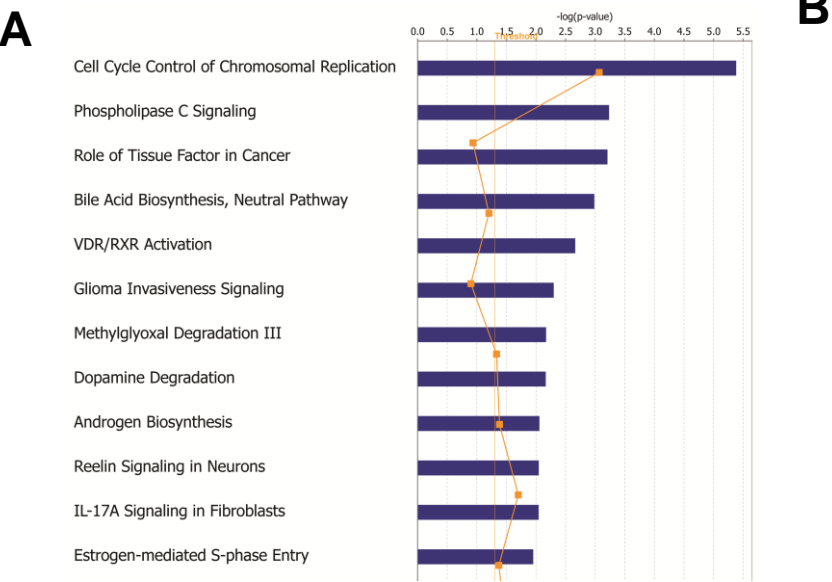


Figure 5



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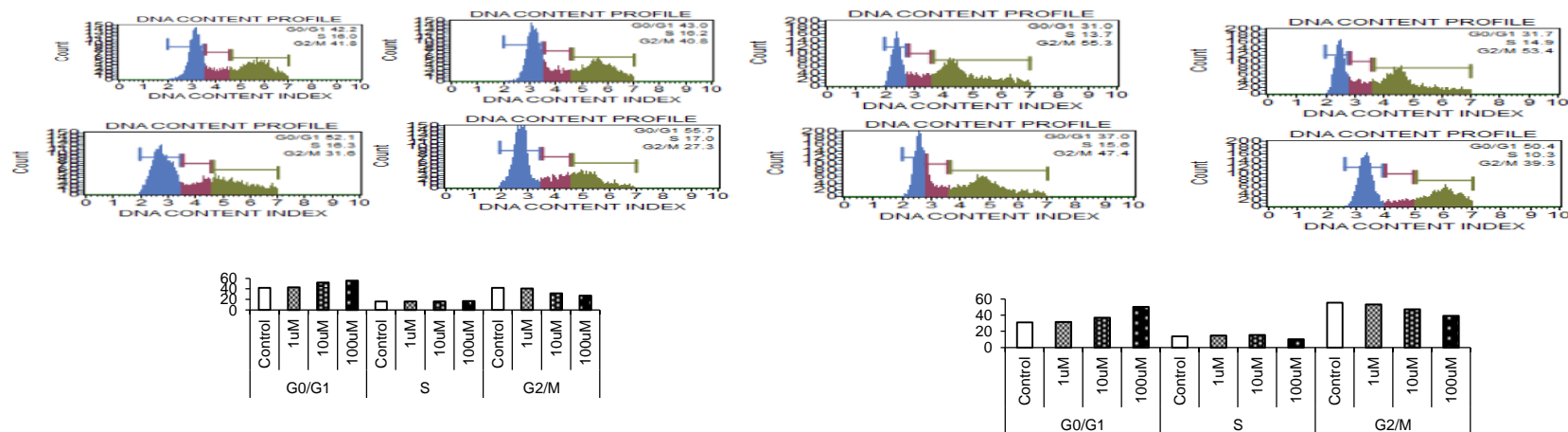
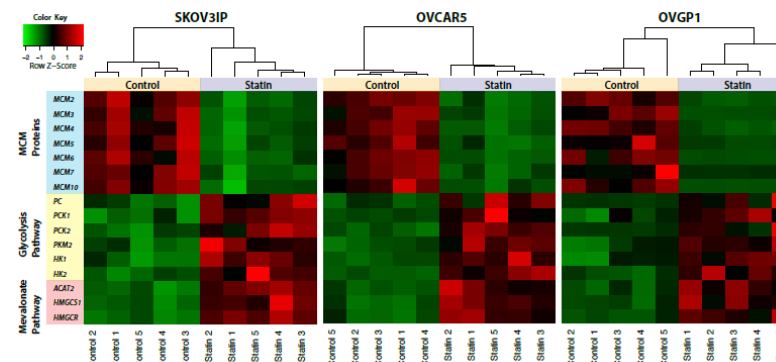
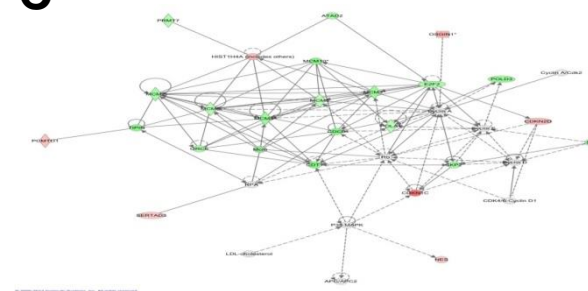
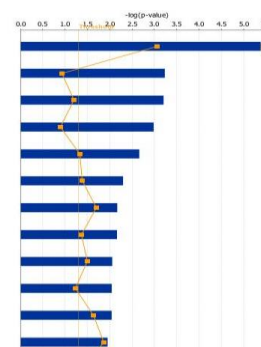
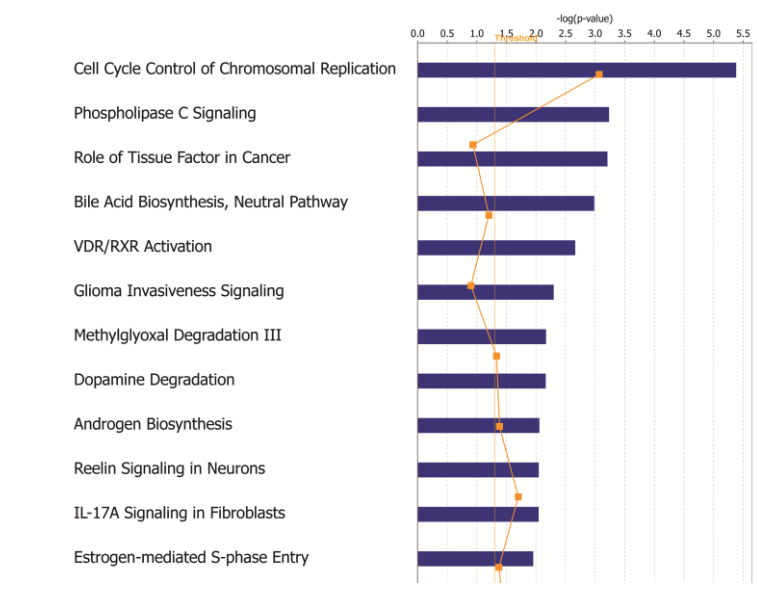
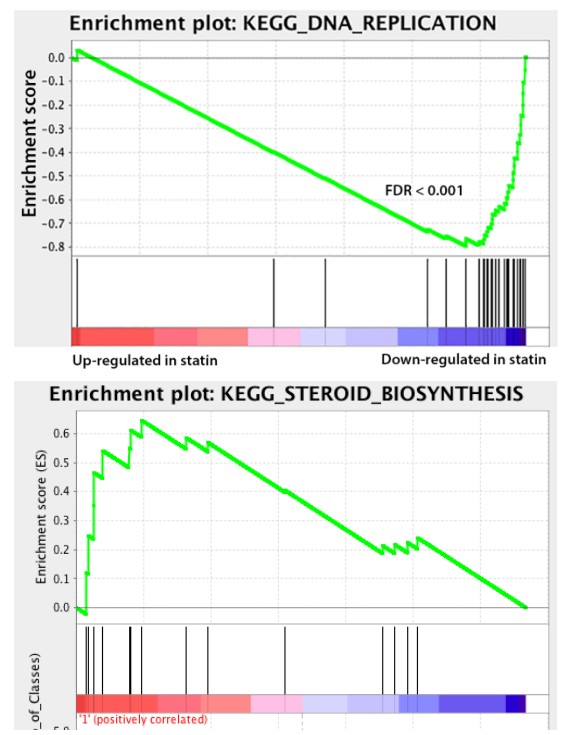


Figure 6

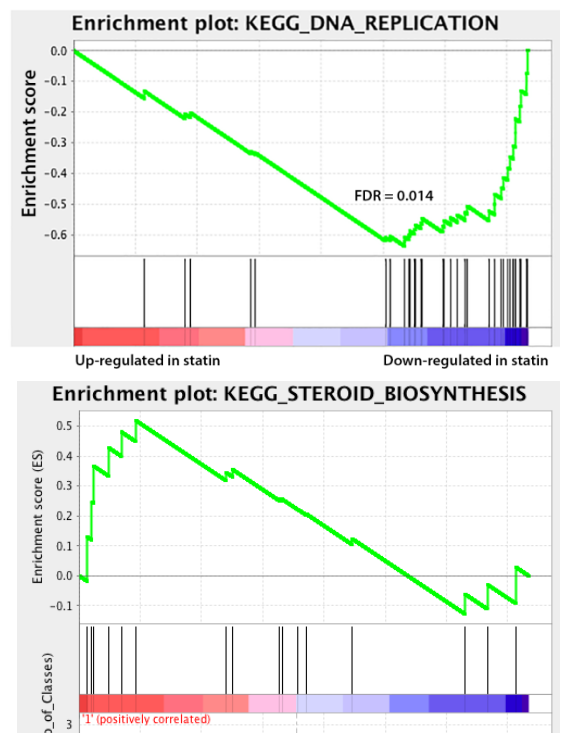
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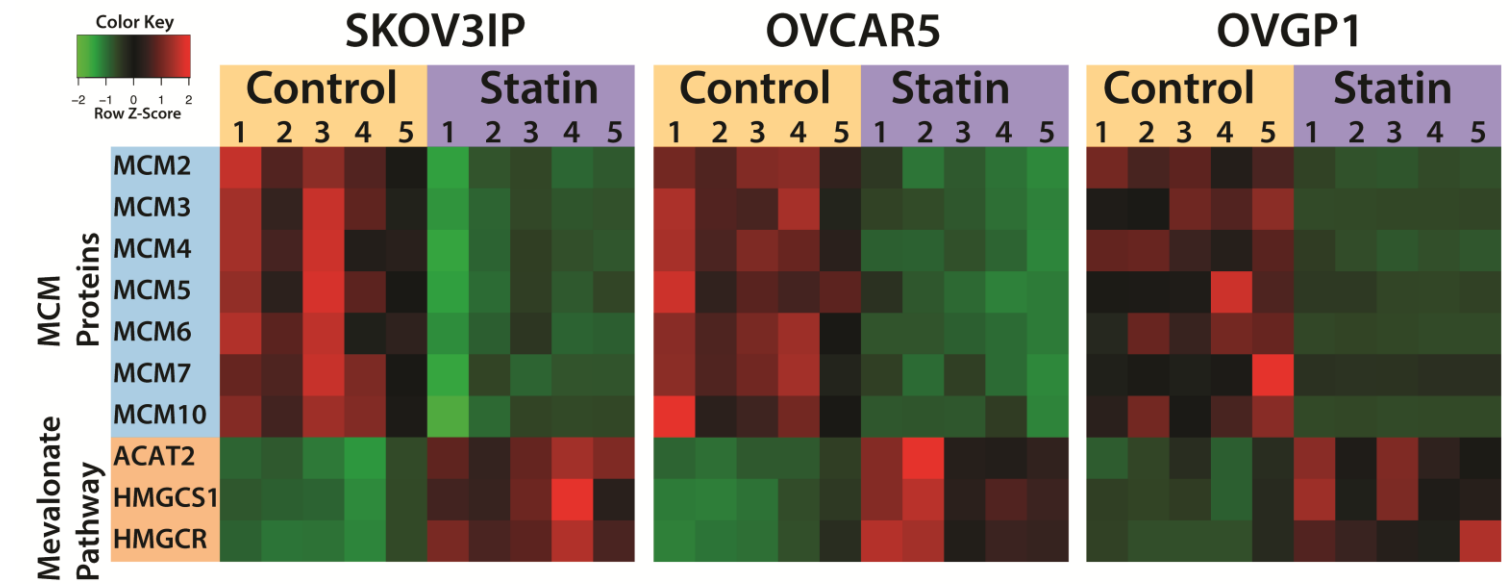
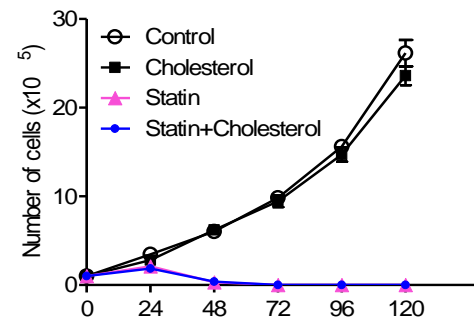
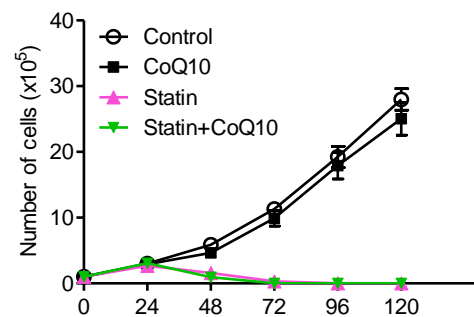
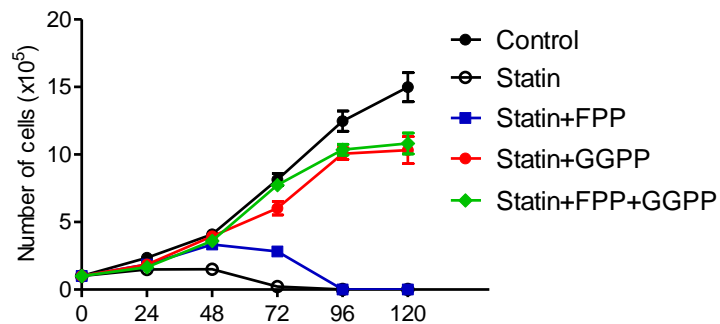
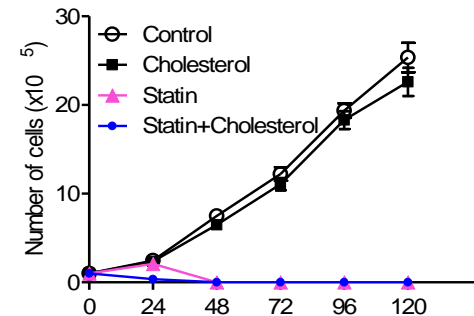
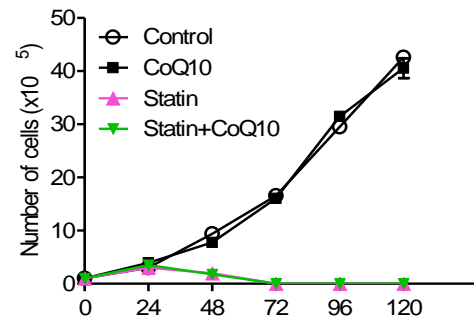
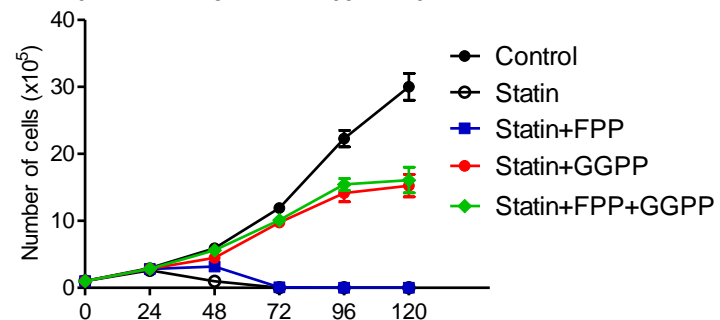


Figure 6

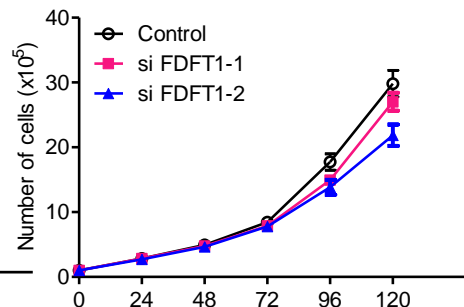
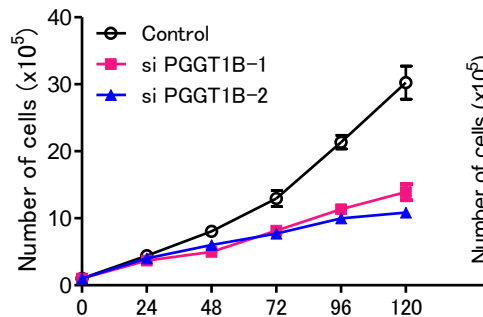
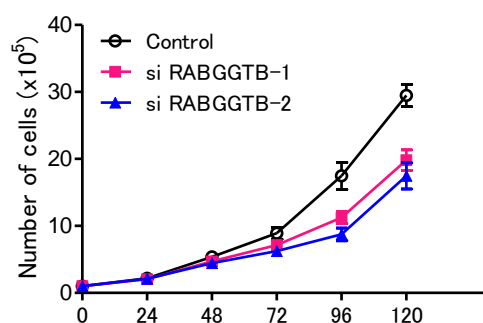
A



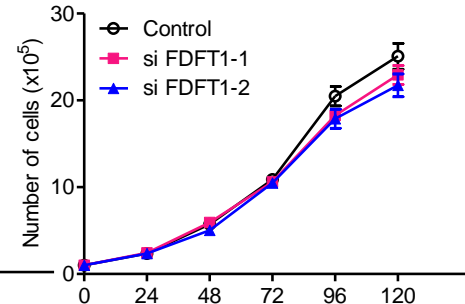
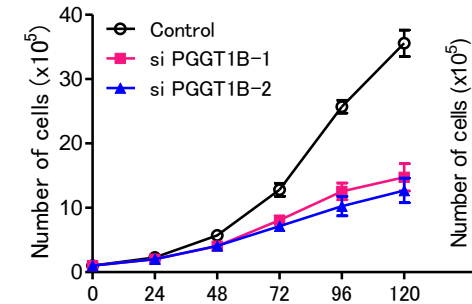
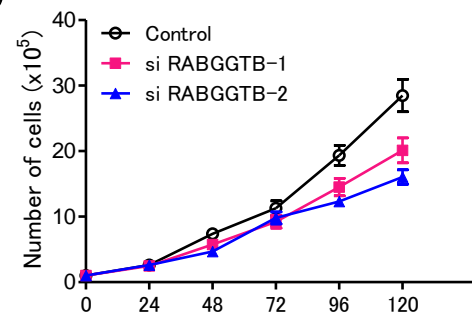
B



C

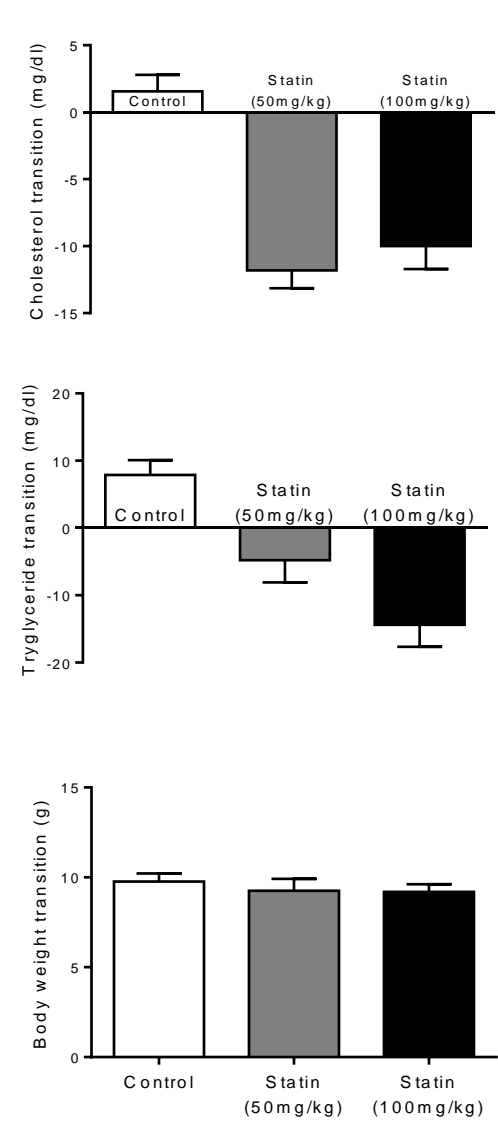


D

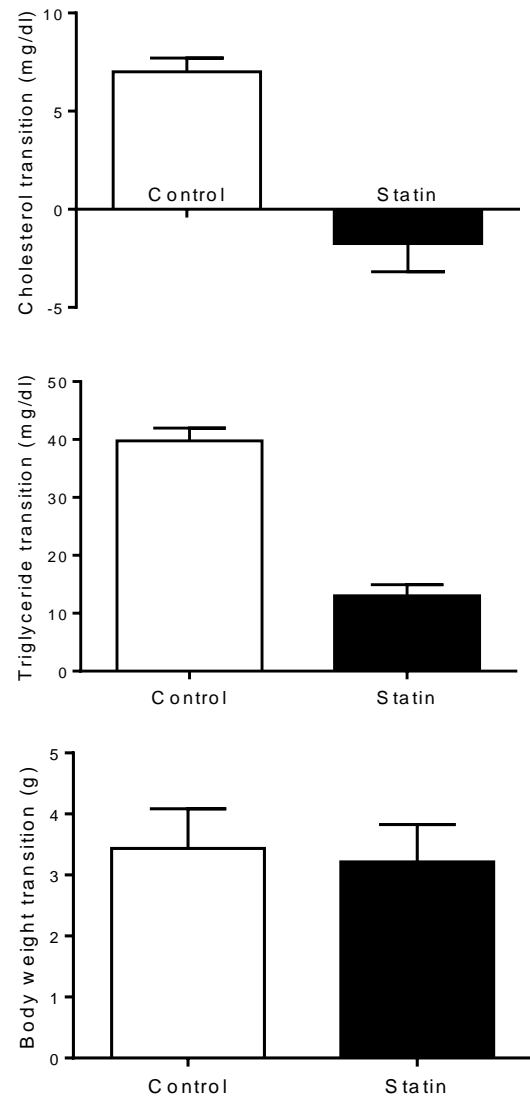


Supplemental Fig. 1

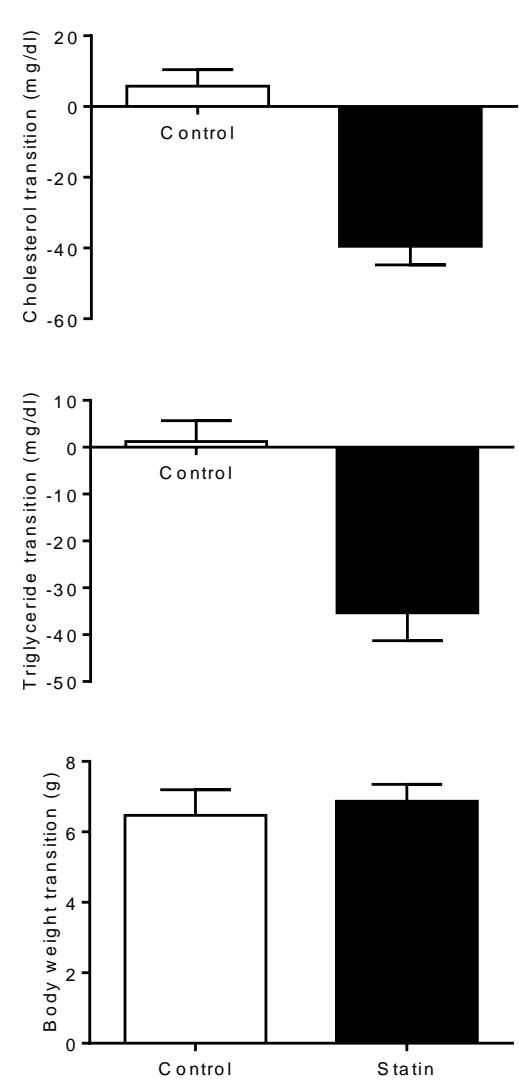
A OVGP1 Mouse Model



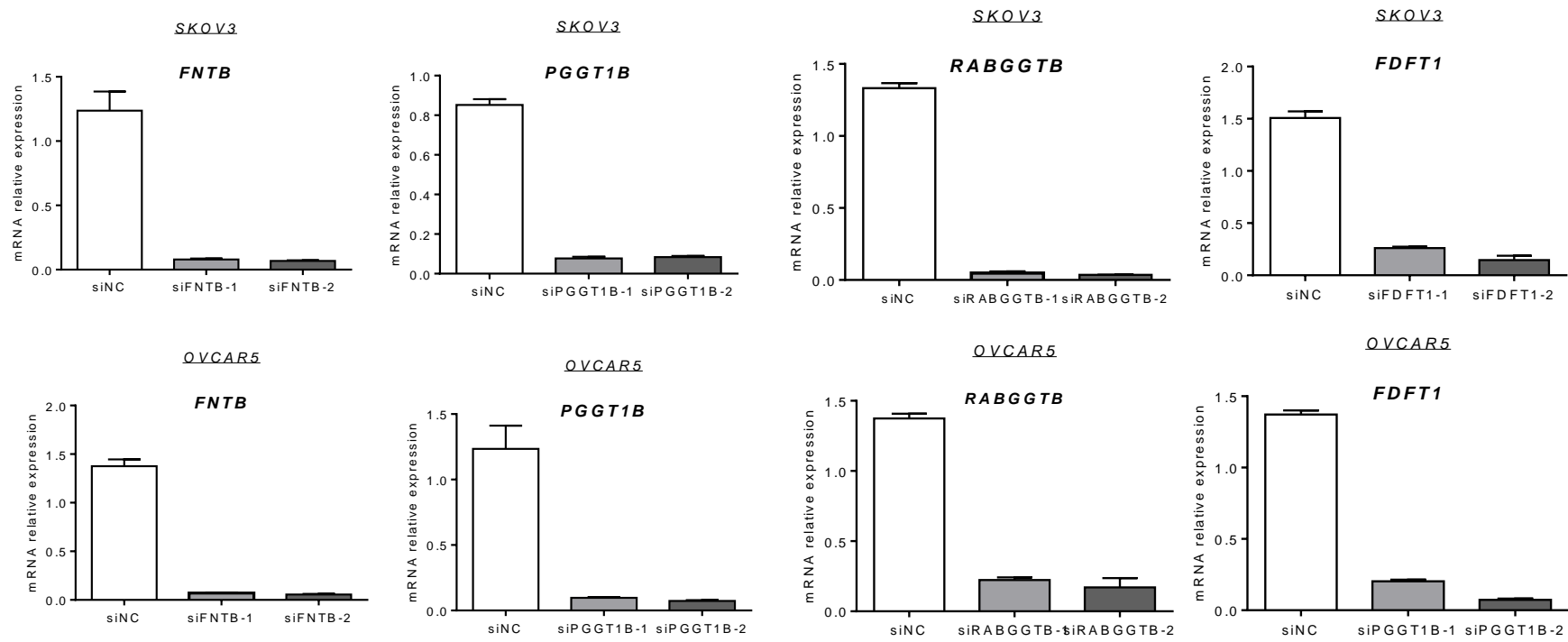
B SKOV3-IP Model



C OVCAR5 Model



Supplemental Fig. 2



Supplemental Figure XX

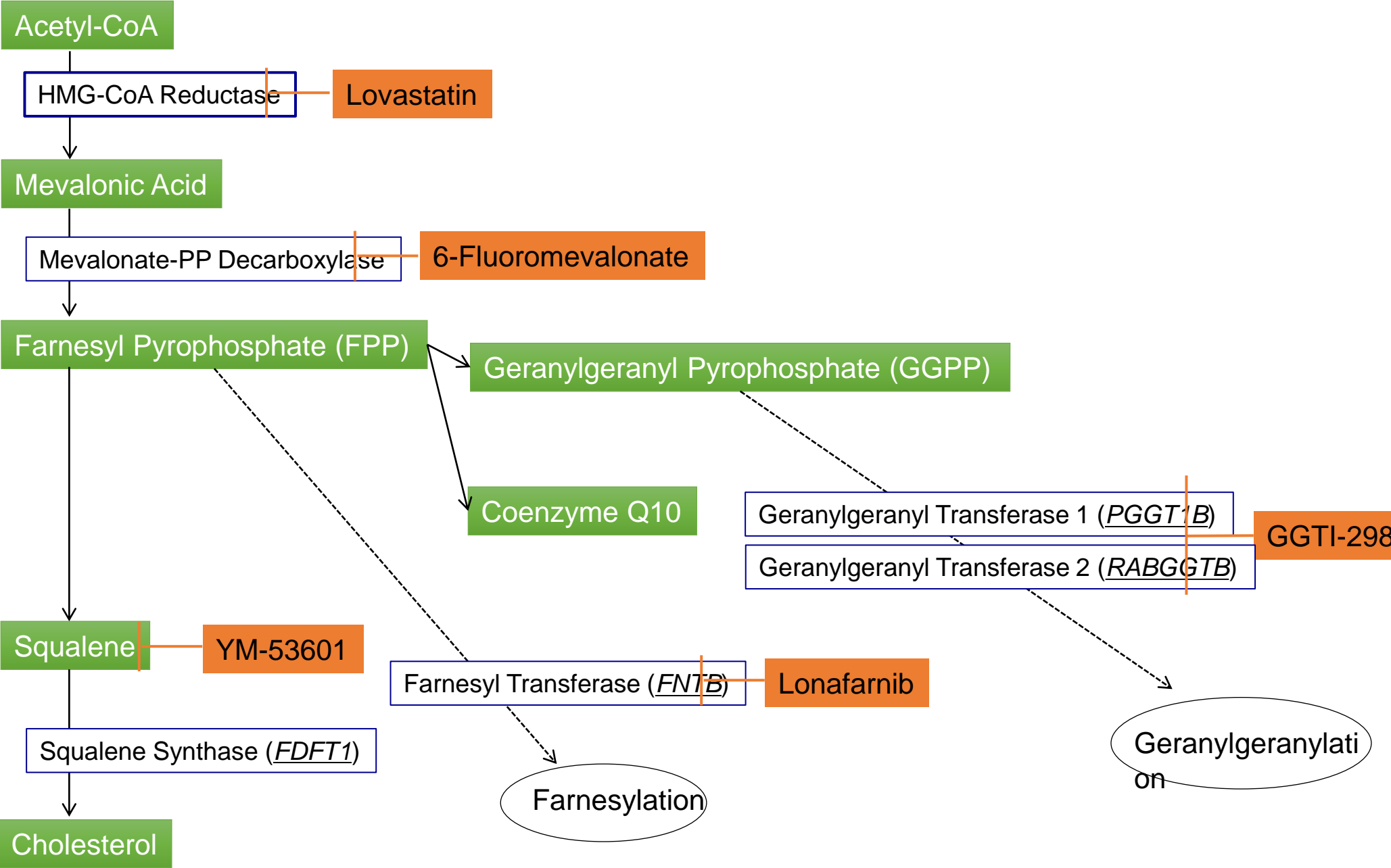
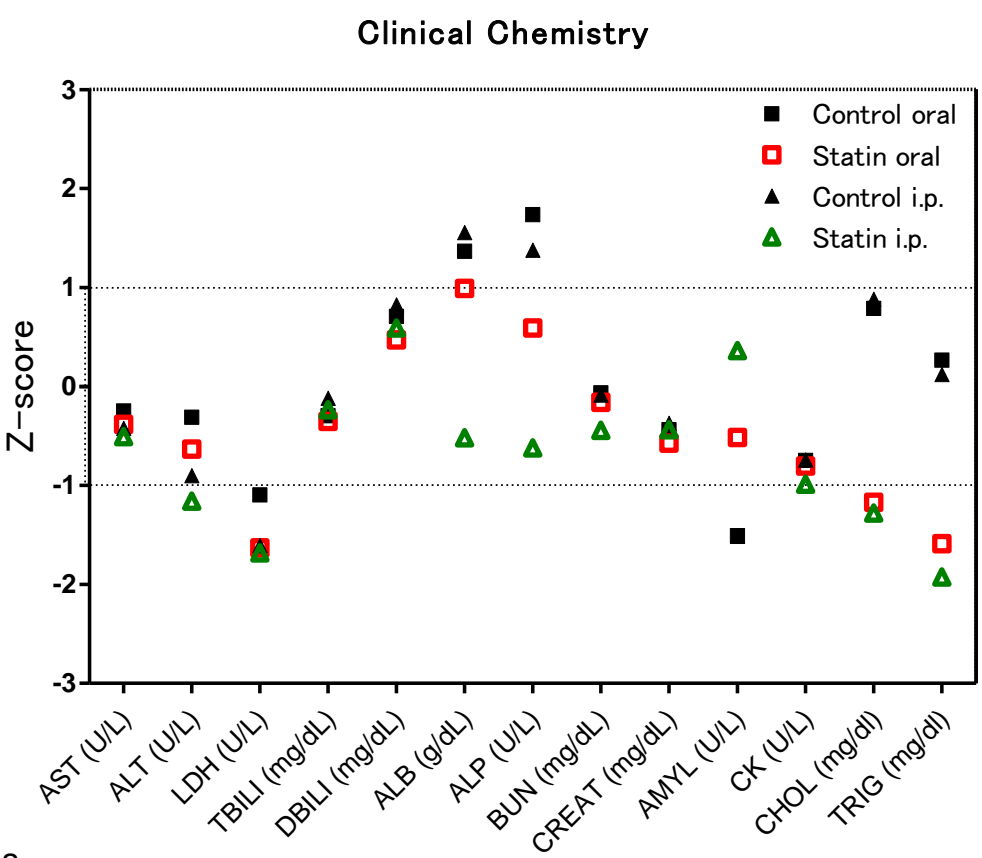
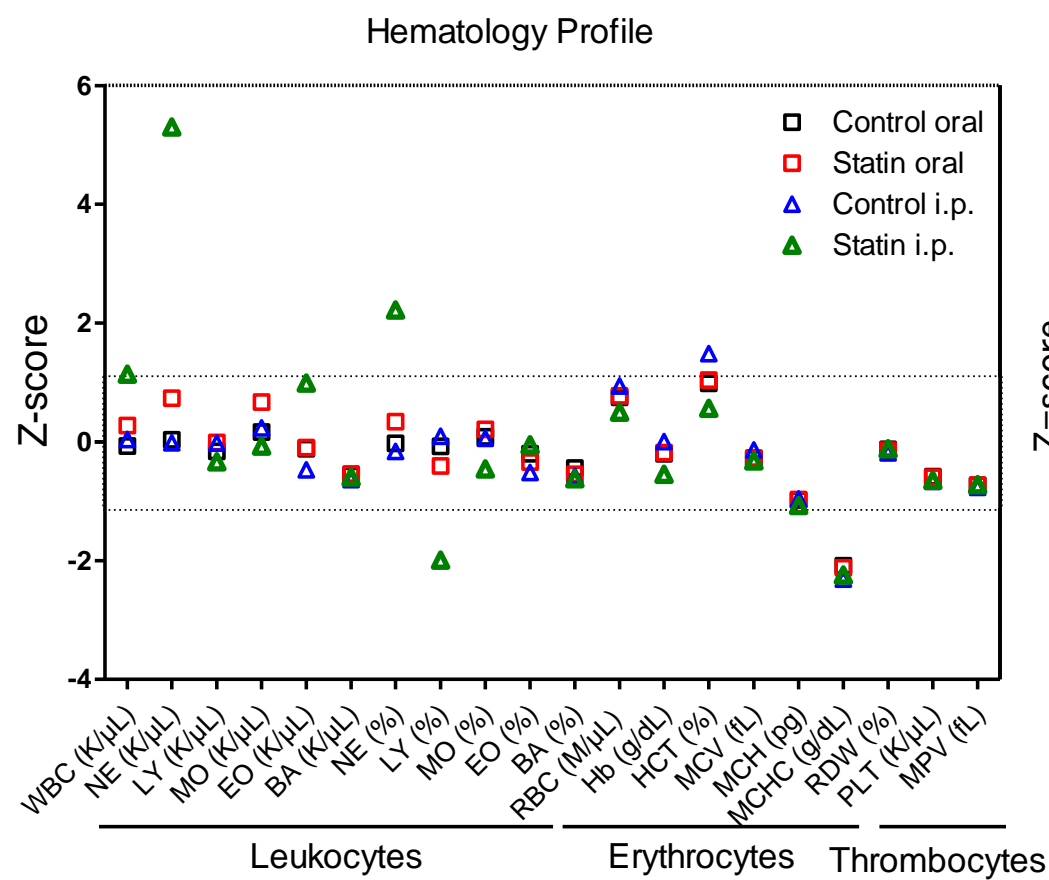


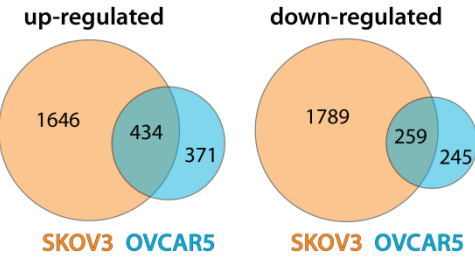
Fig. 5



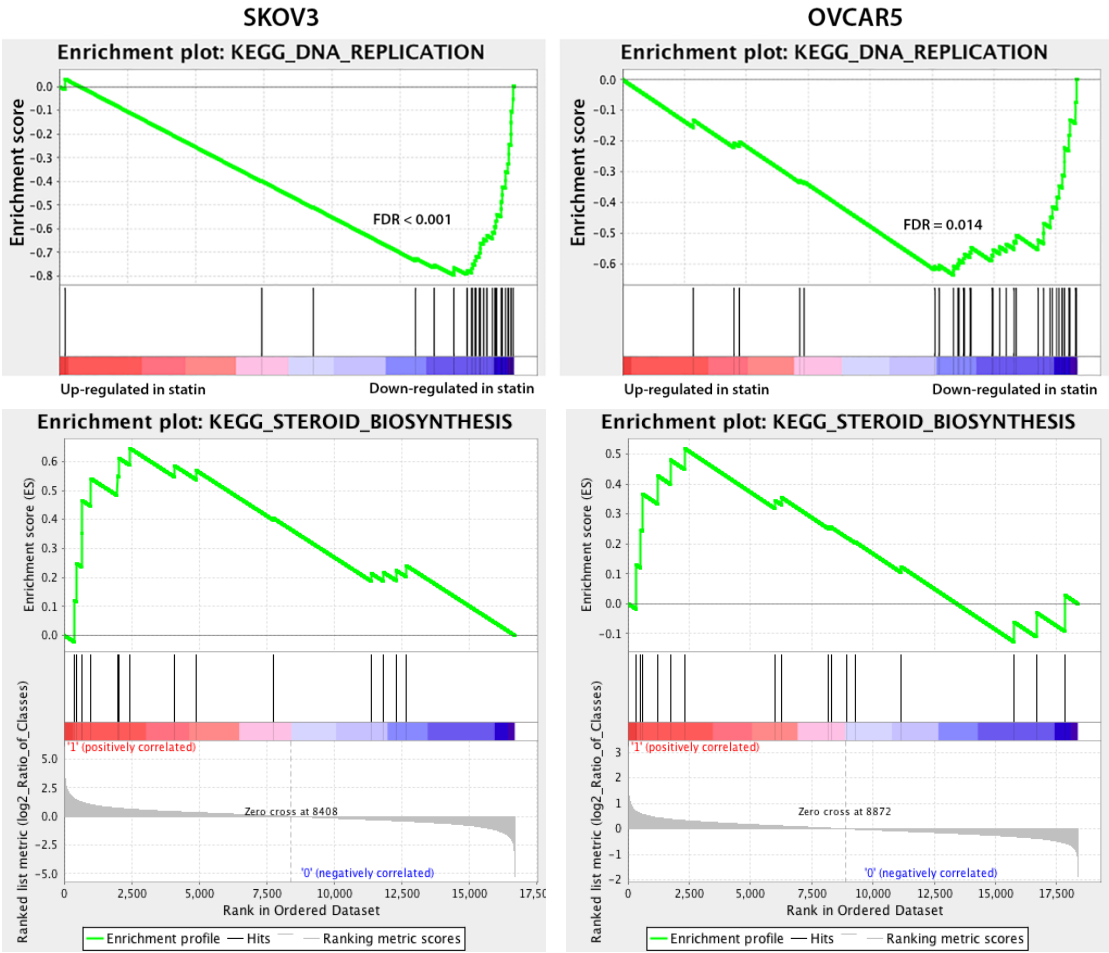
Supple. 3

A

Overlapped Differentially Expressed Genes



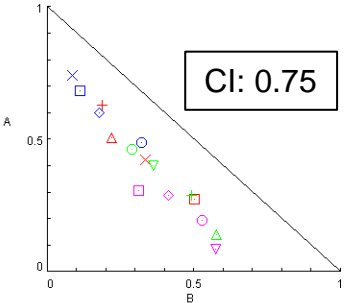
B



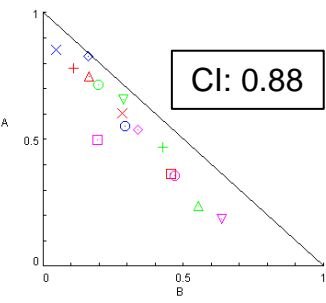
A

SKOV3

A; Lovastatin, B; Paclitaxel

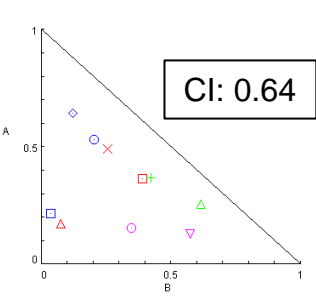


A; Lovastatin, B; Carboplatin

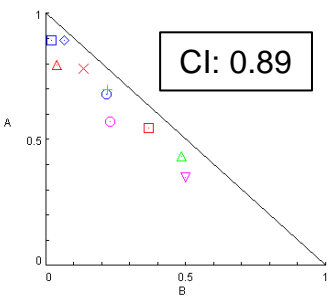


OVCAR5

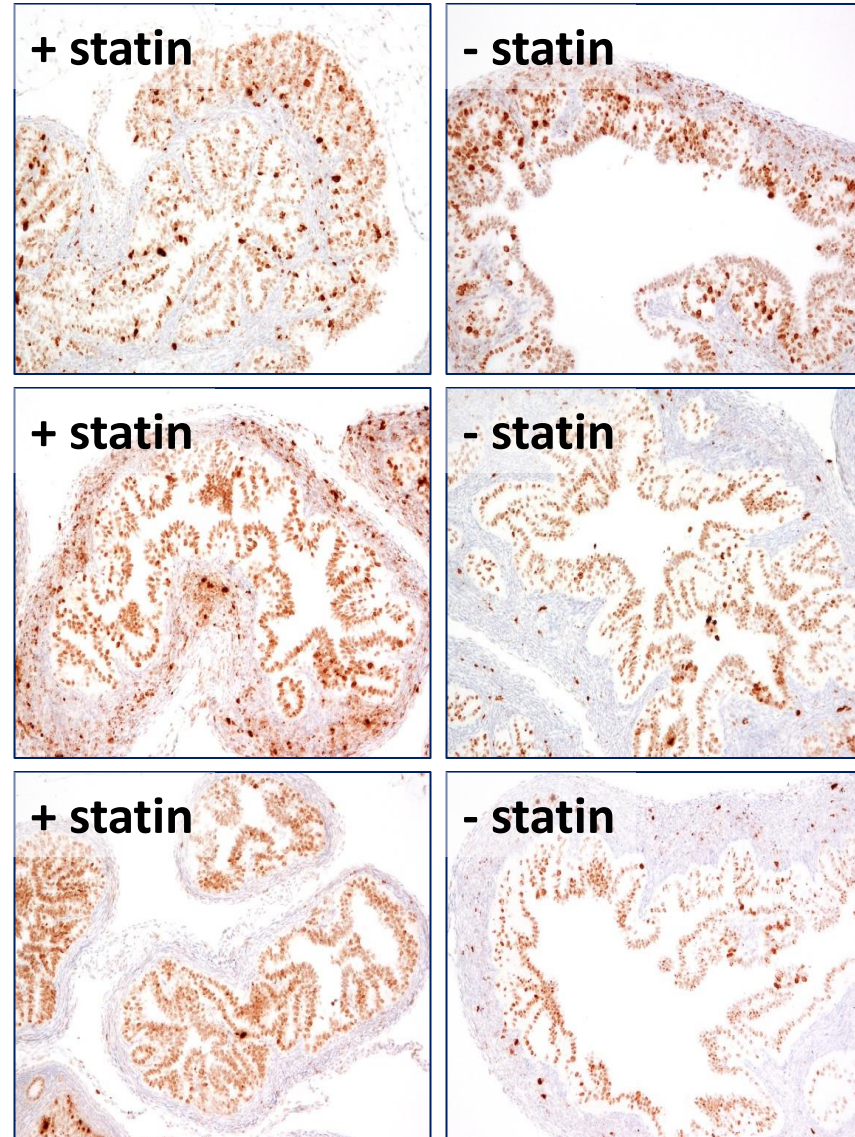
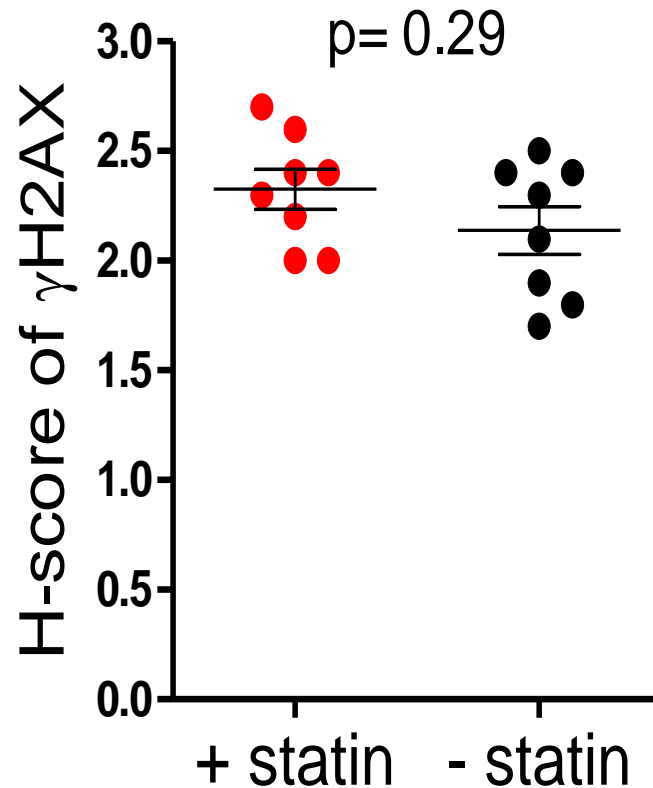
A; Lovastatin, B; Paclitaxel



A; Lovastatin, B; Carboplatin



Statin treatment does not affect γ H2AX immunoreactivity in fallopian tube



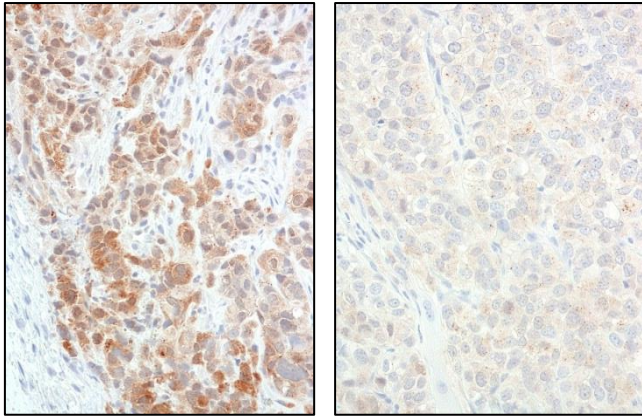
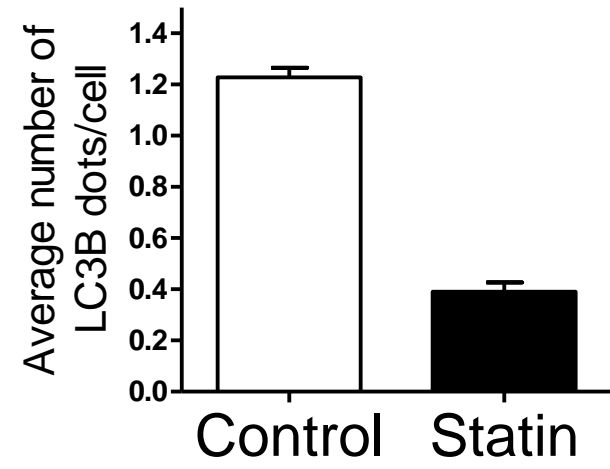
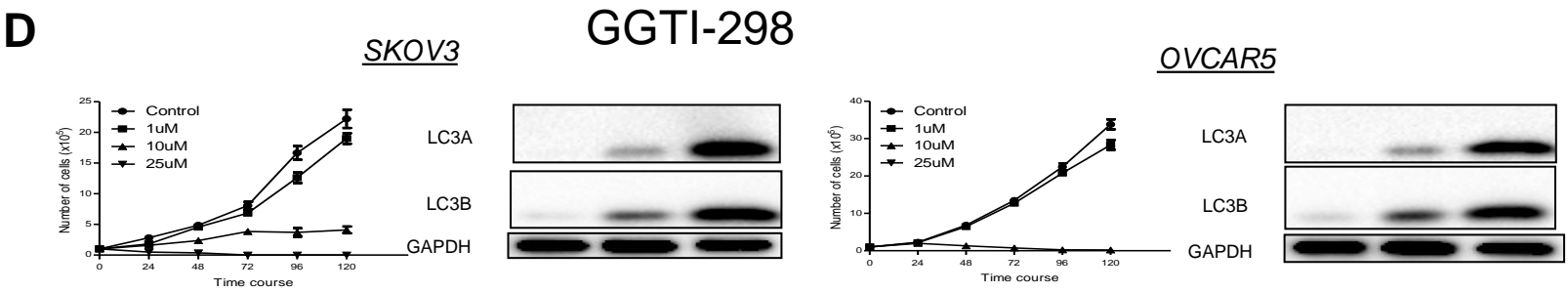
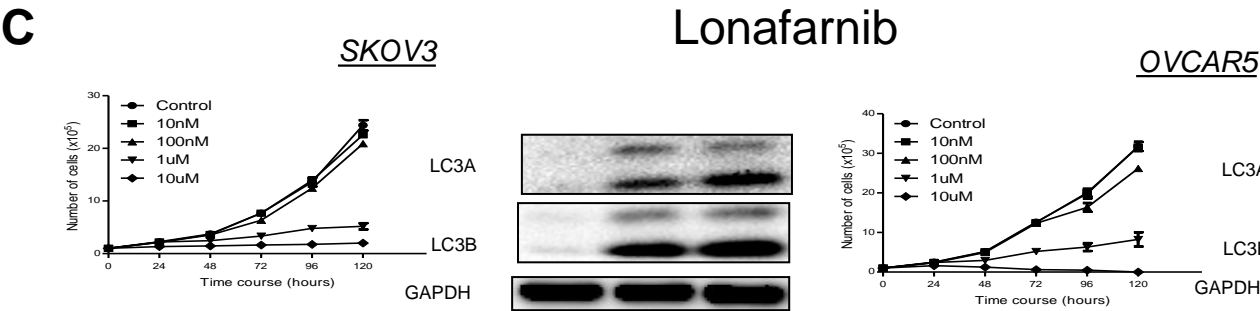
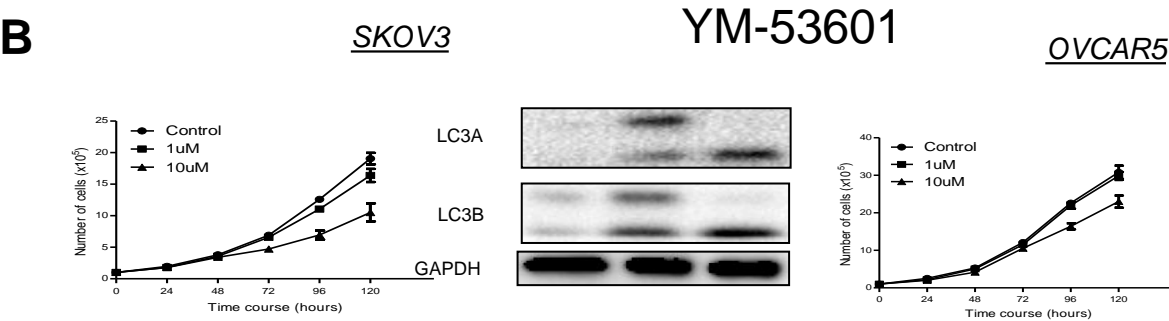
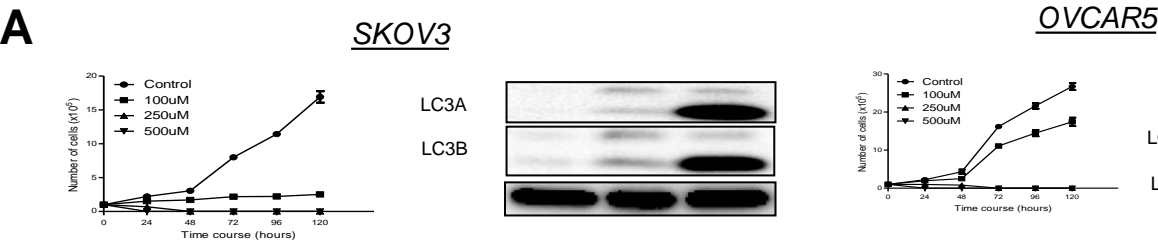
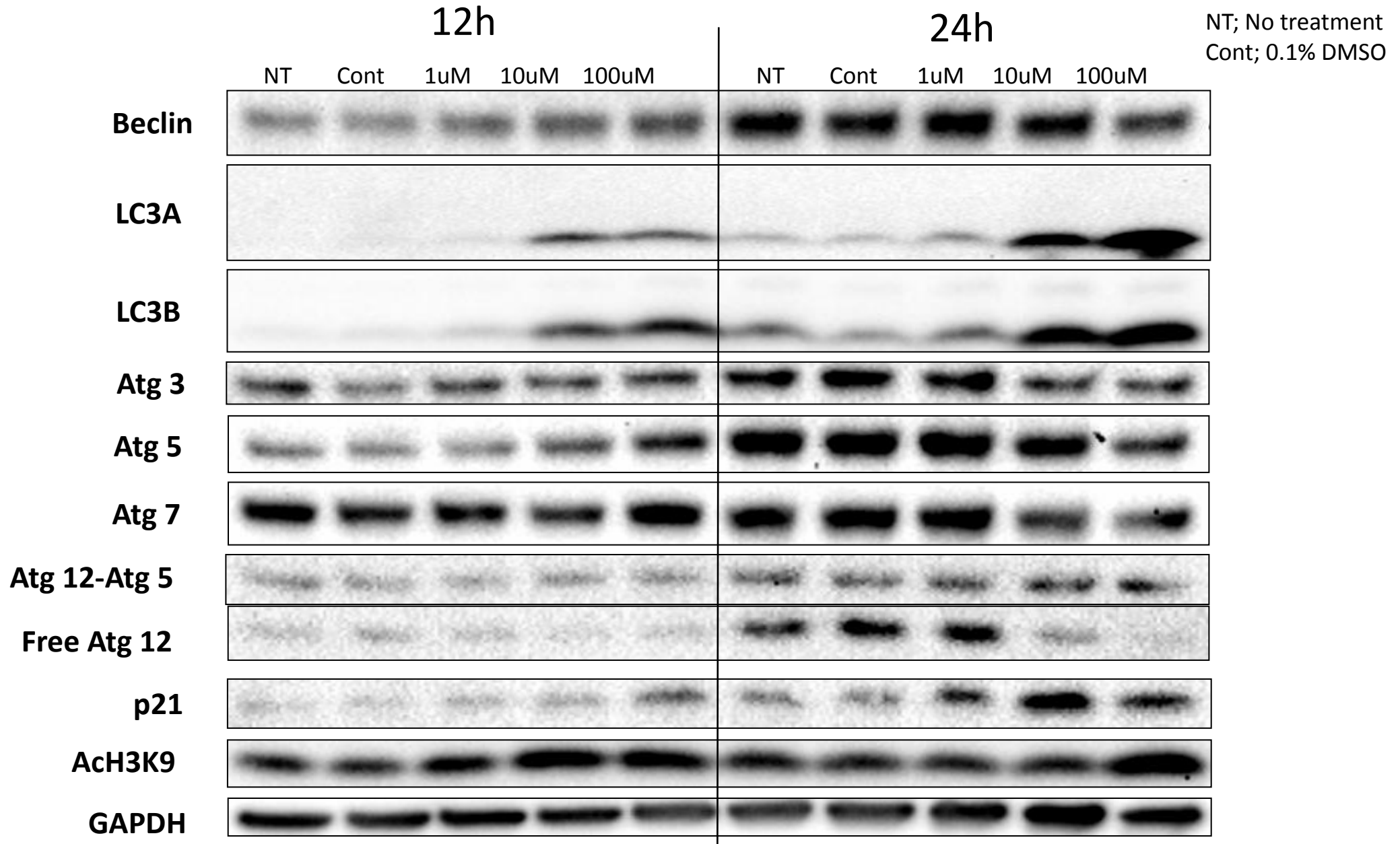


Figure 6

6-Fluoromevalonate



Autophagy in OVCAR5



24h

48h

NT

Cont

1uM

10uM

100uM

NT

Cont

1uM

10uM

100uM

Beclin

LC3A

LC3B

Atg 3

Atg 5

Atg 7

Atg 12-Atg 5

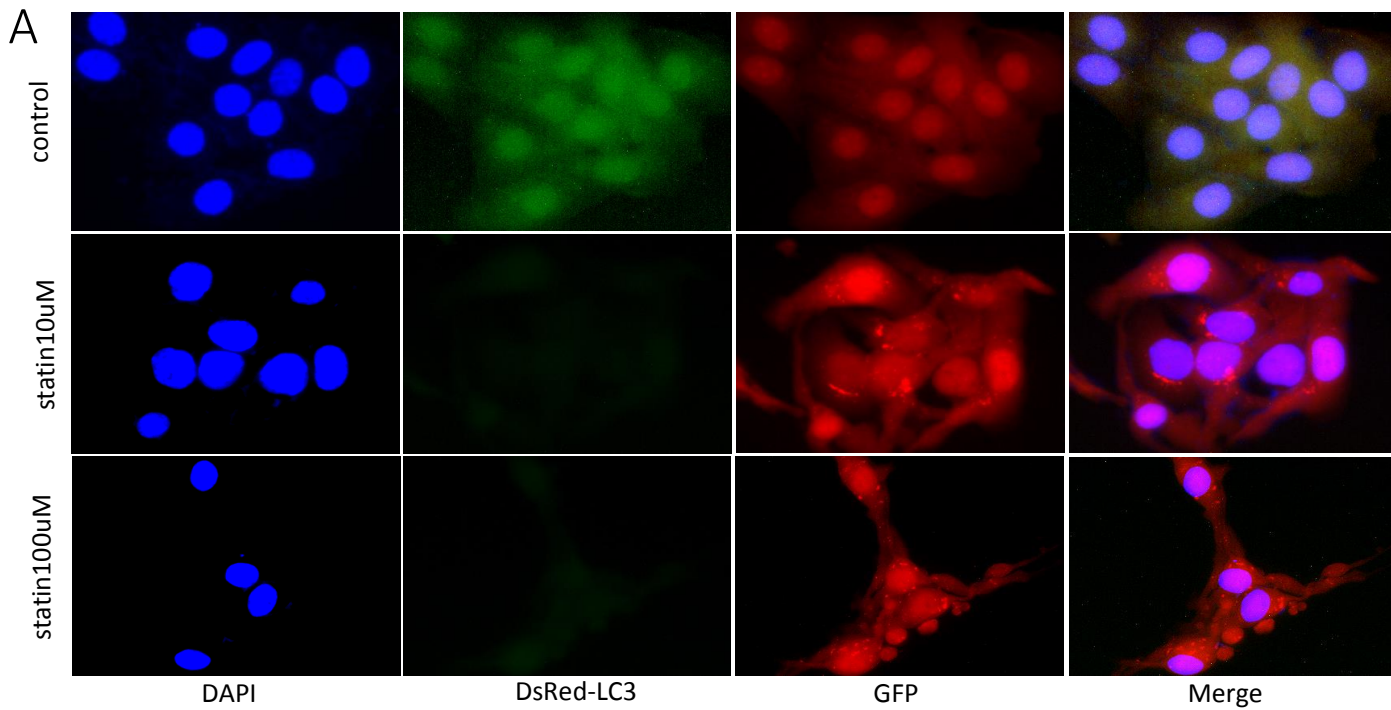
Free Atg 12

p21

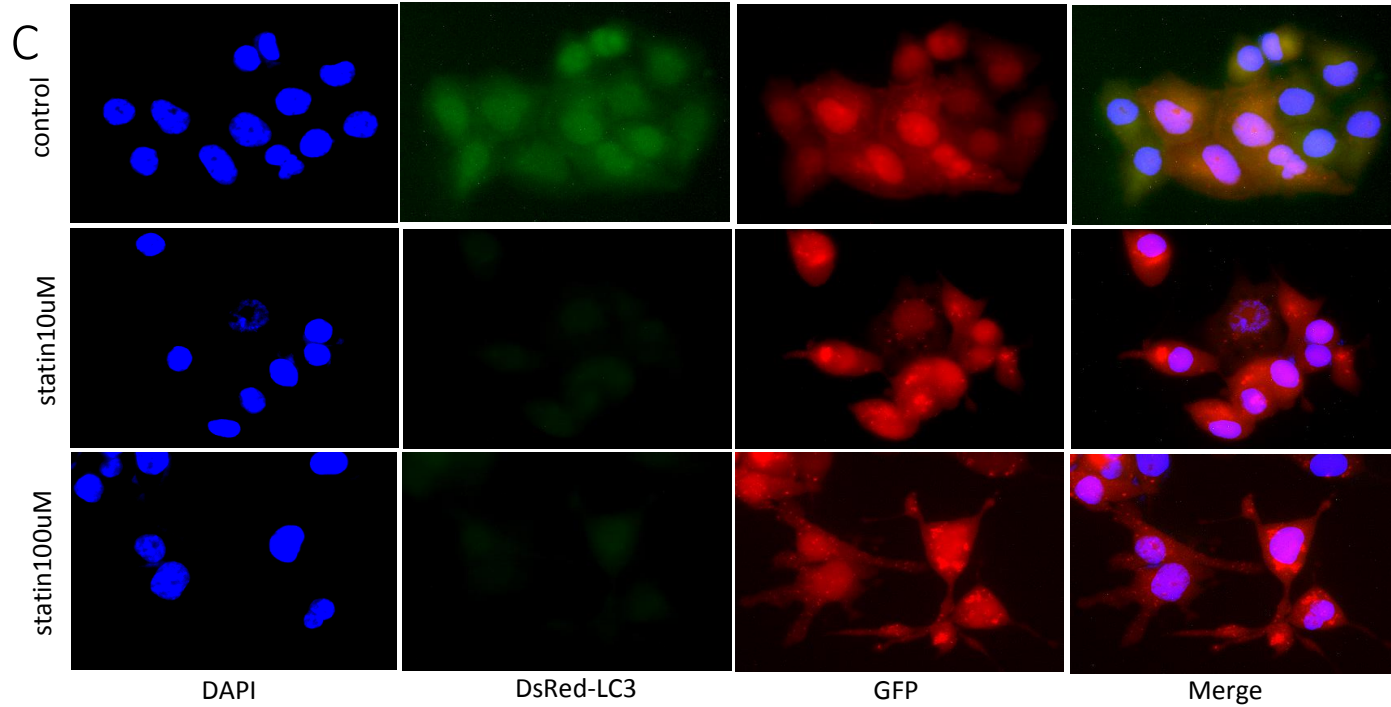
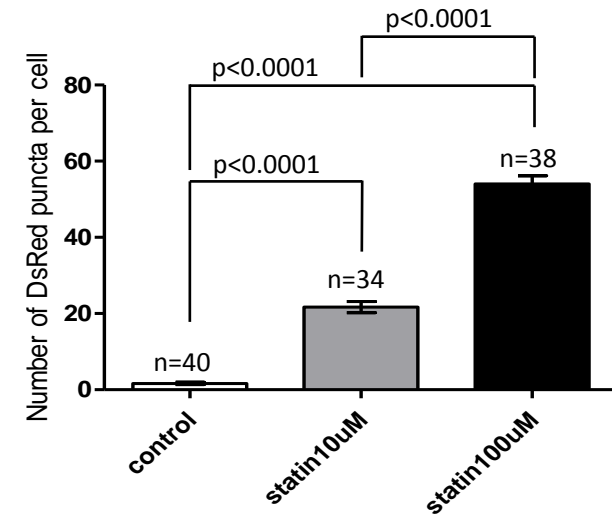
AcH3K9

GAPDH

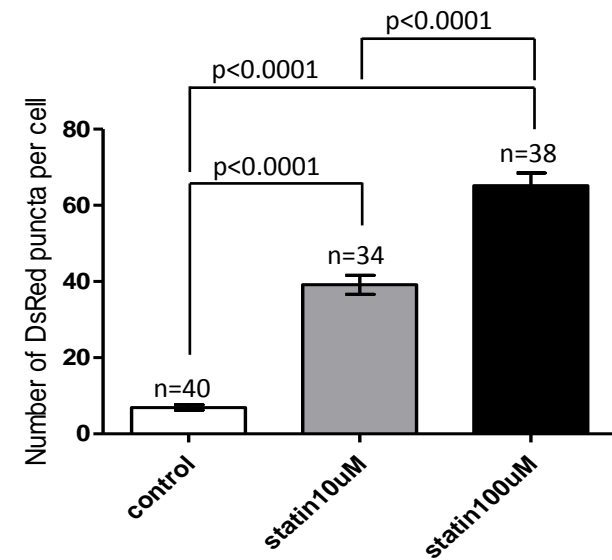




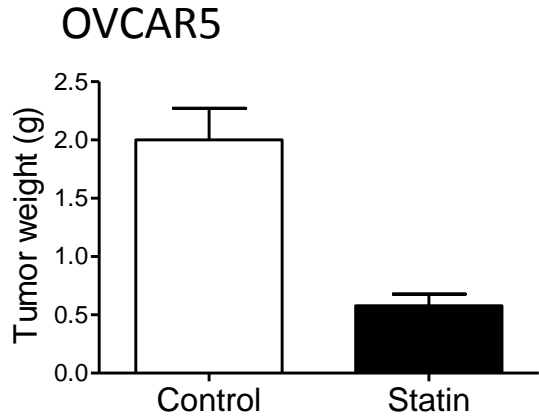
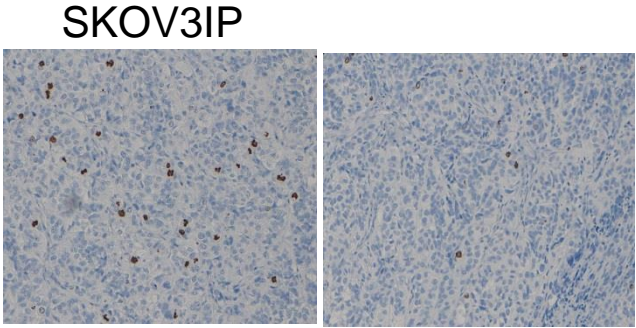
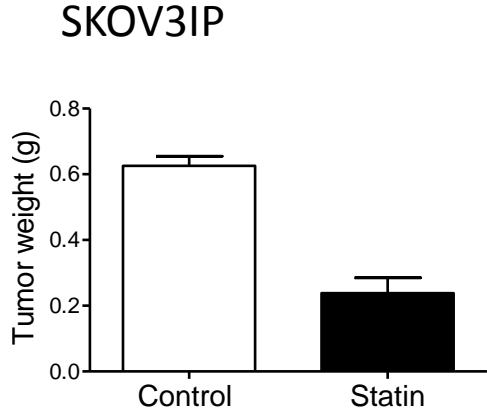
B



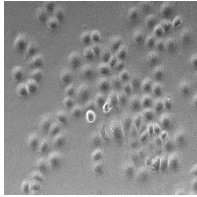
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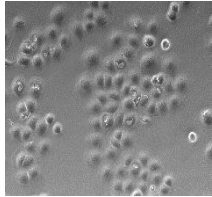
Supplementary Figures



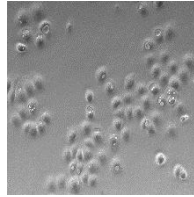
OVCAR3



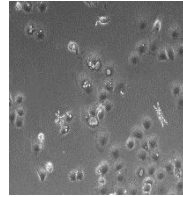
Control



1 uM

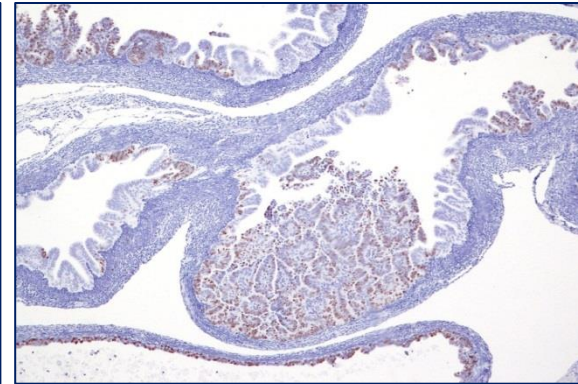
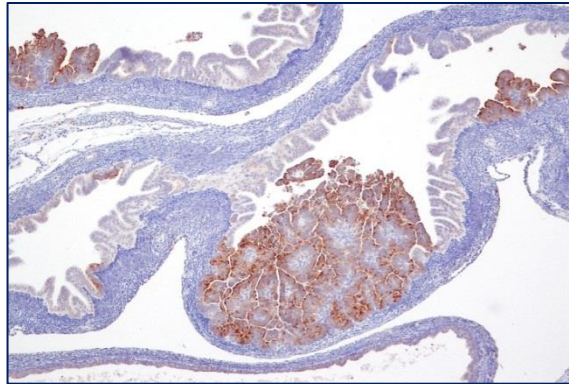
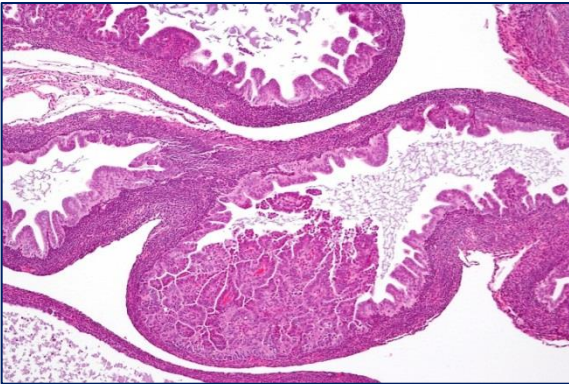
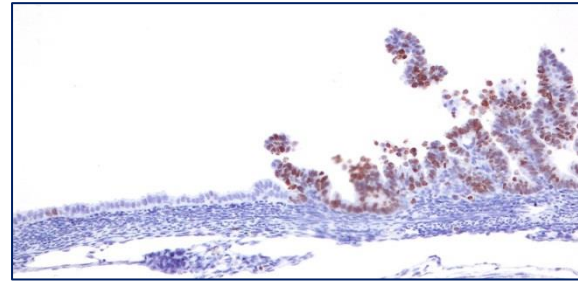
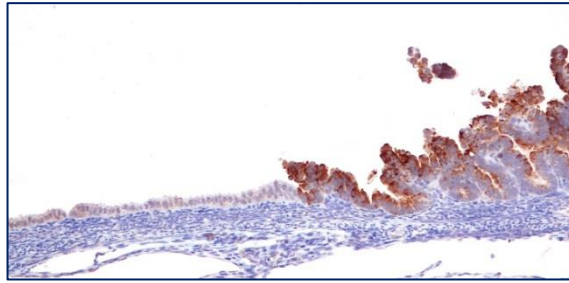
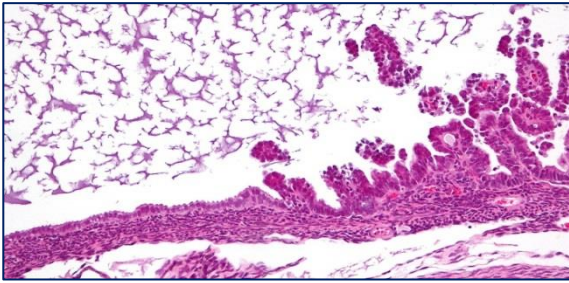


10 uM



100 uM

Koba: could you show OVCAR5 photo instead?



Supplemental Figure

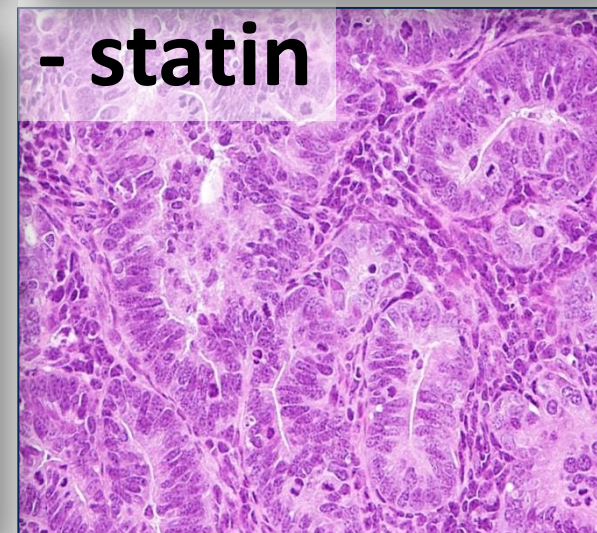
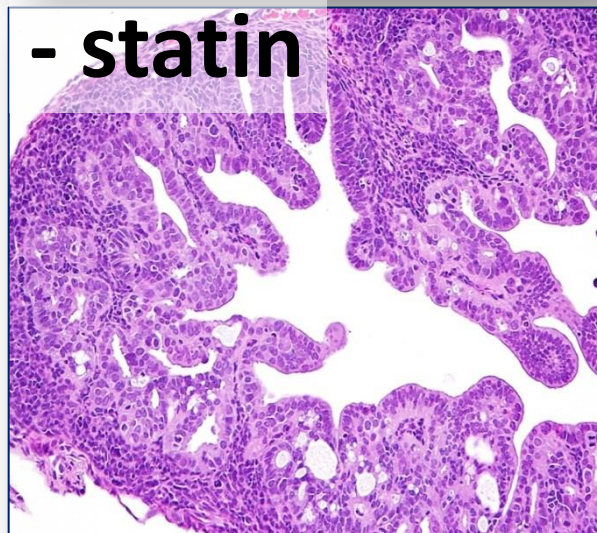
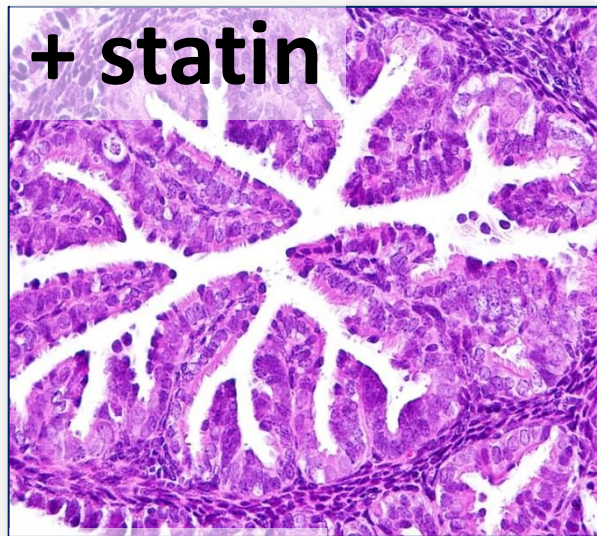
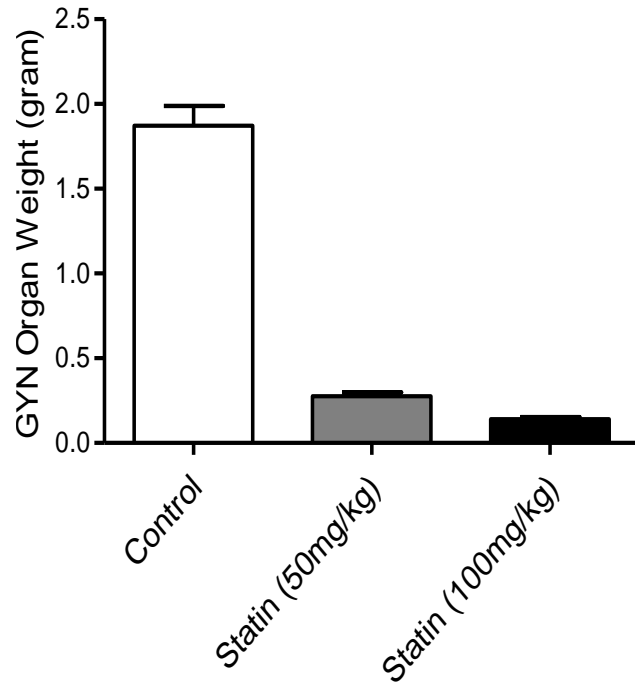
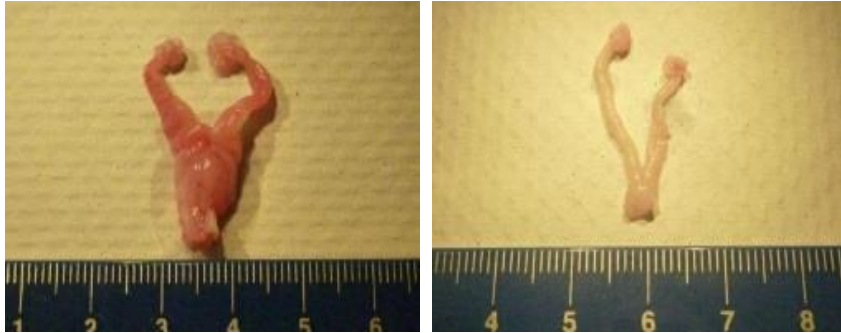


Figure 2

Lovastatin (dose?)

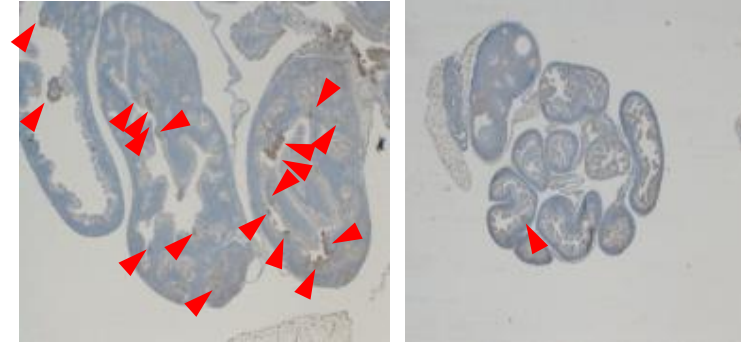
A

Control



B

Control



Lovastatin (dose?)
Retake picture by
le-Ming

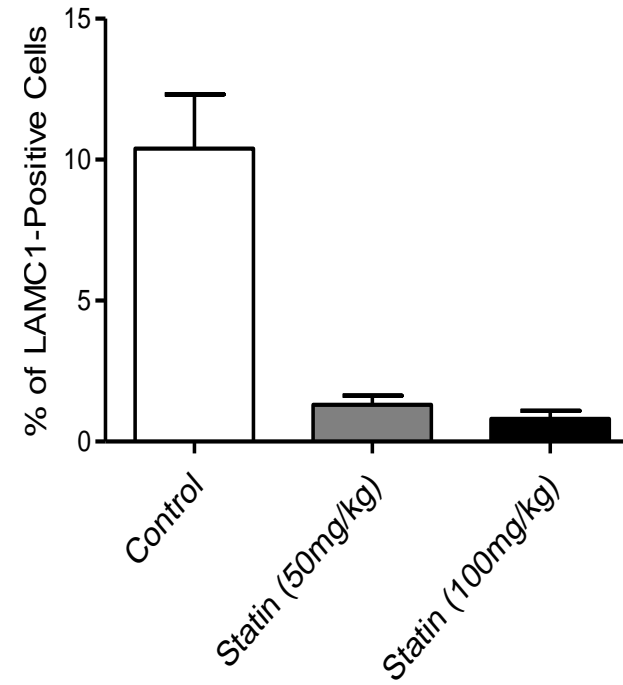


Figure 5

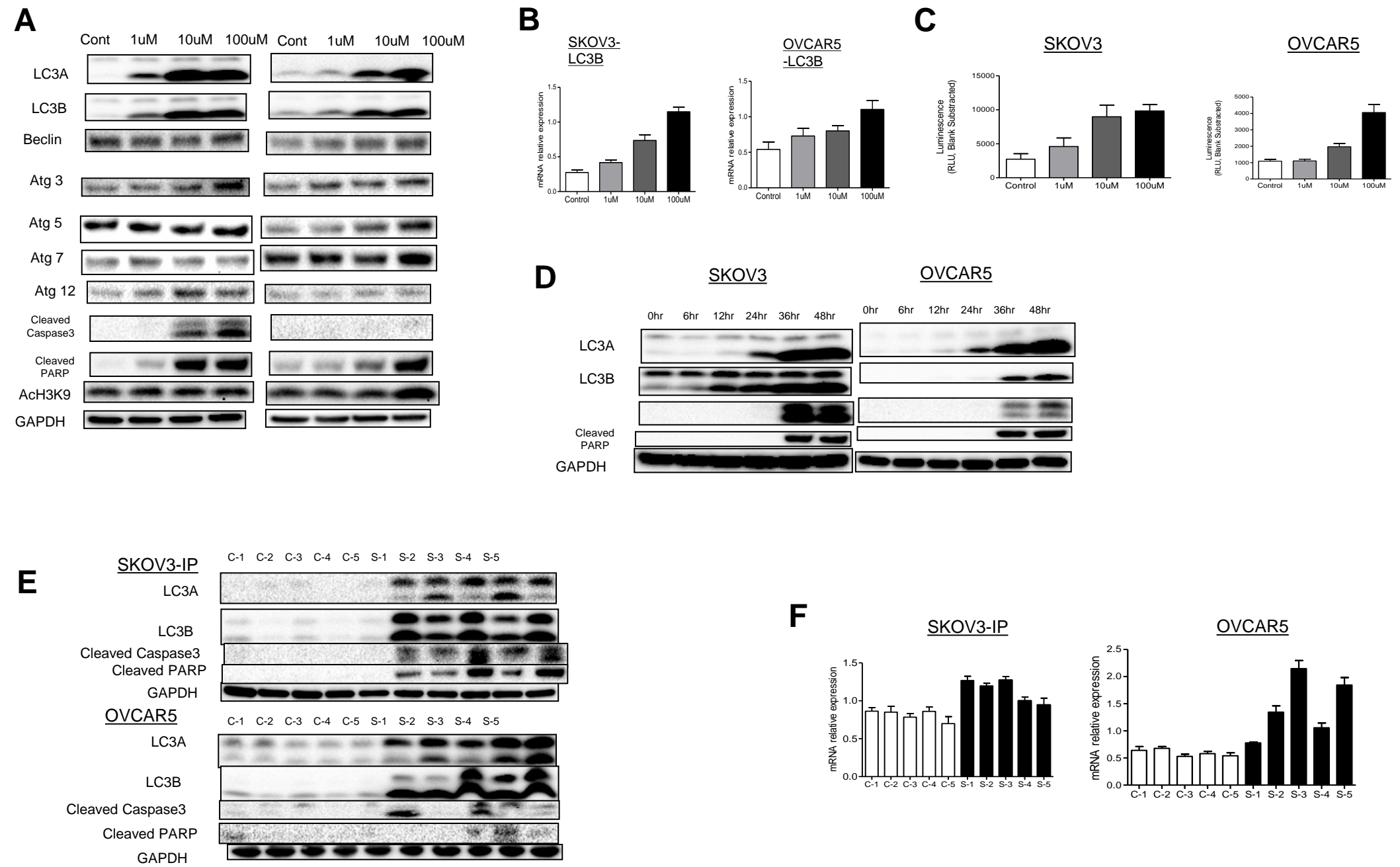
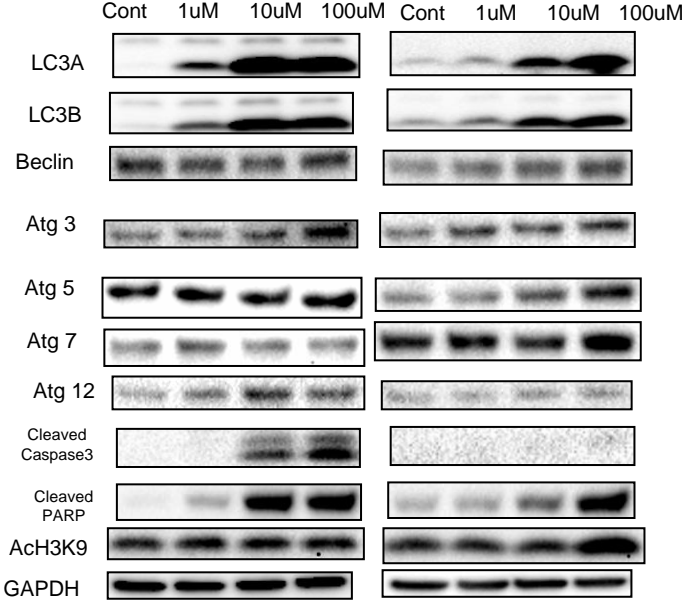


Figure 5

A



B

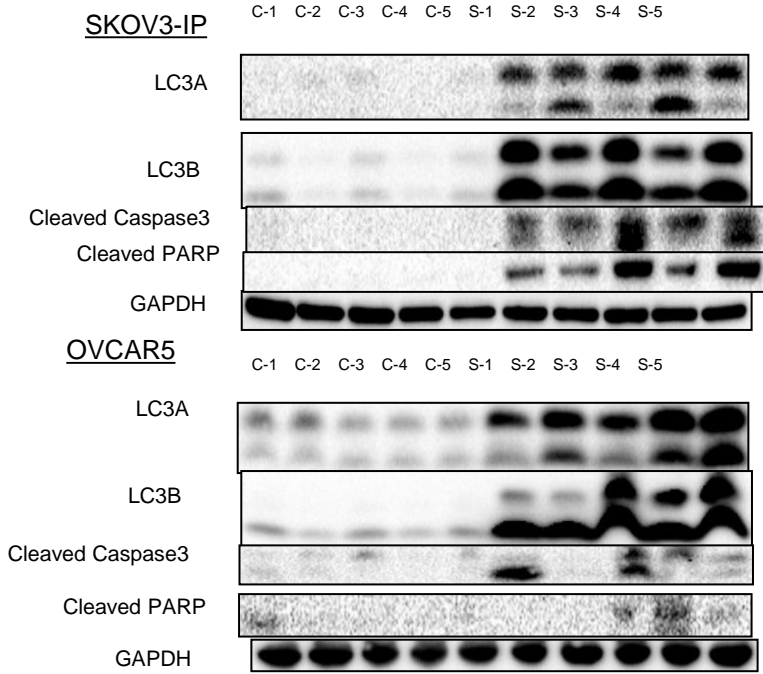
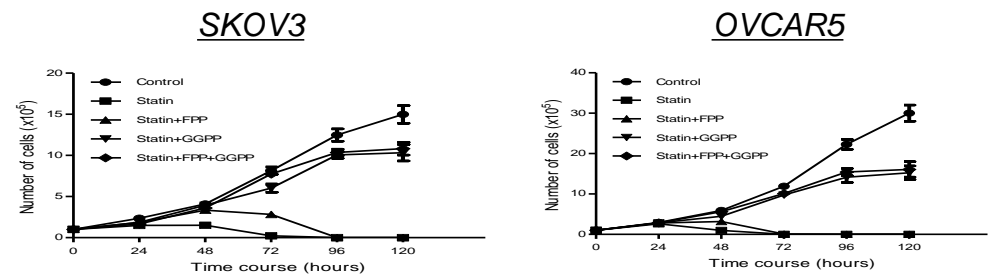
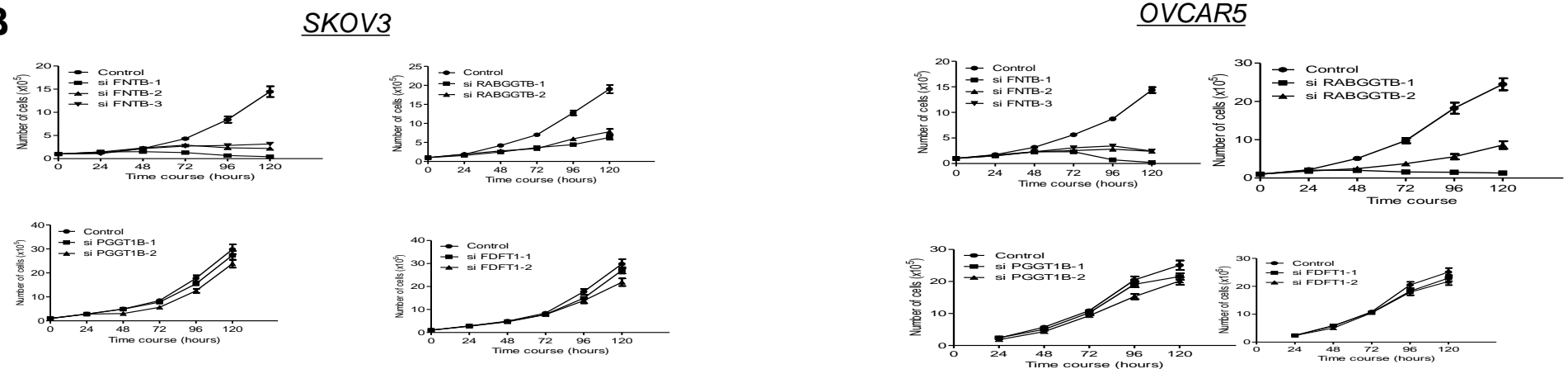


Figure 7

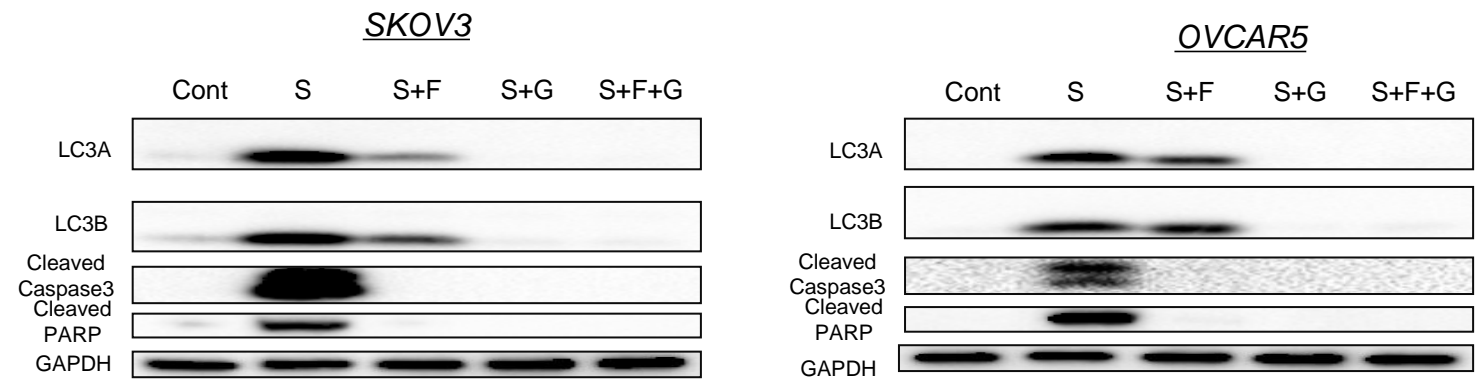
A



B



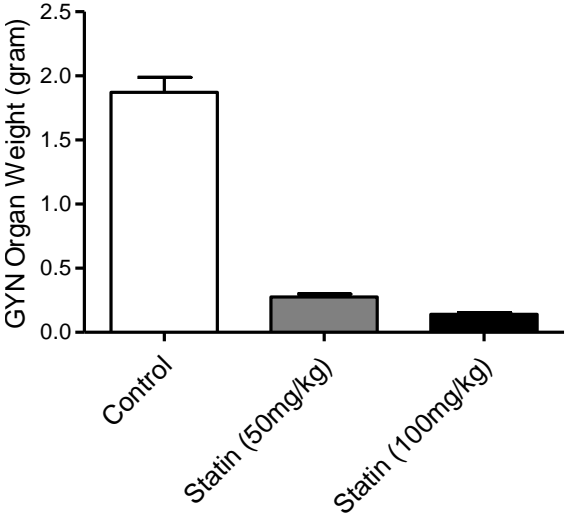
C



S; Statin (10uM)
S+F; Statin(10uM) + FPP(25uM)
S+G; Statin (10uM) + GGPP (25uM)
S+F+G; Statin (10uM) + FPP(25uM) + GGPP(25uM)

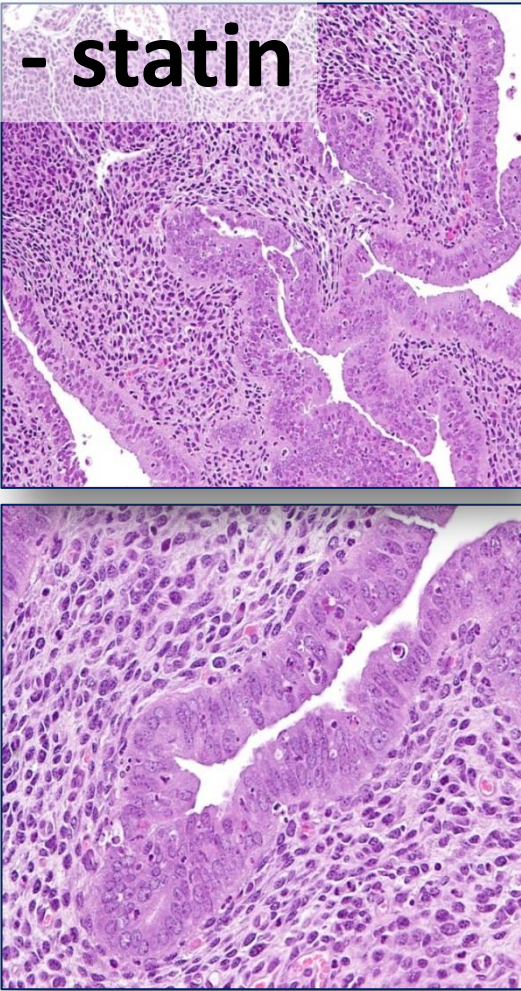
Figure 2

A



Control Statin (50 mg/kg)

B



C

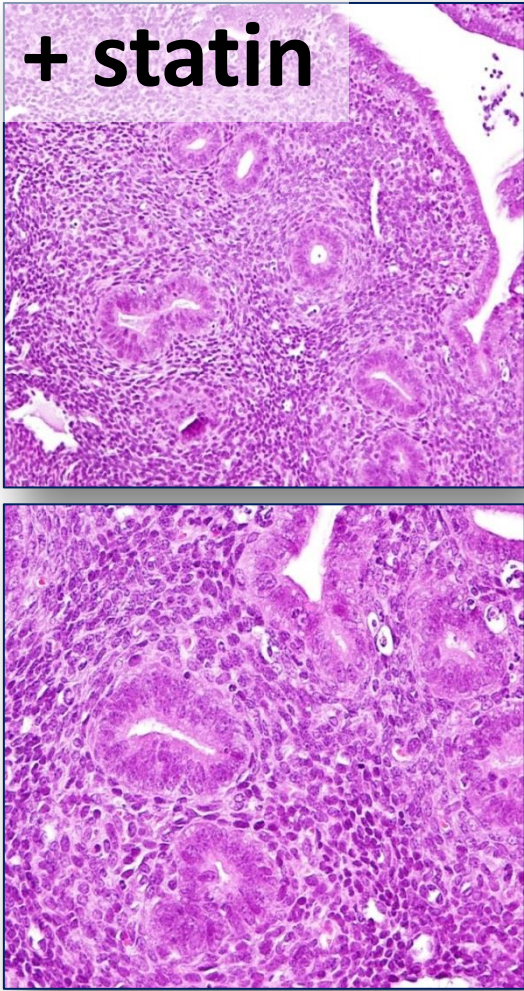
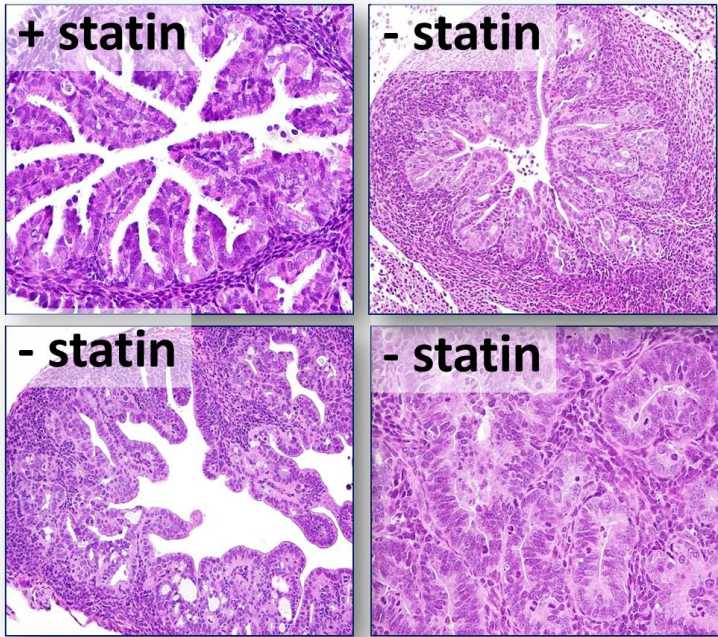
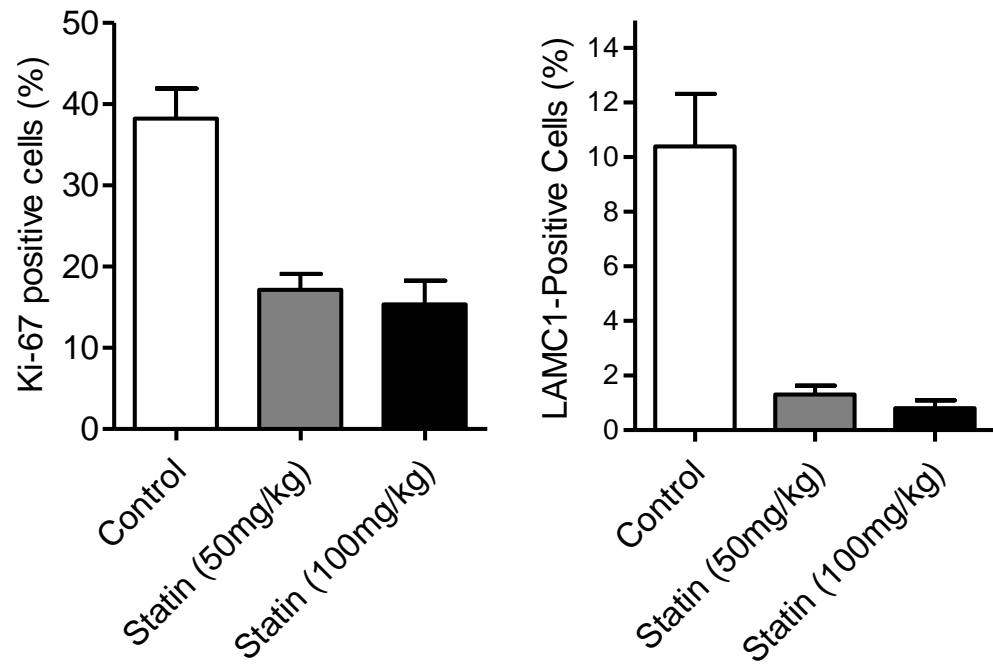


Figure 3

A



B



C

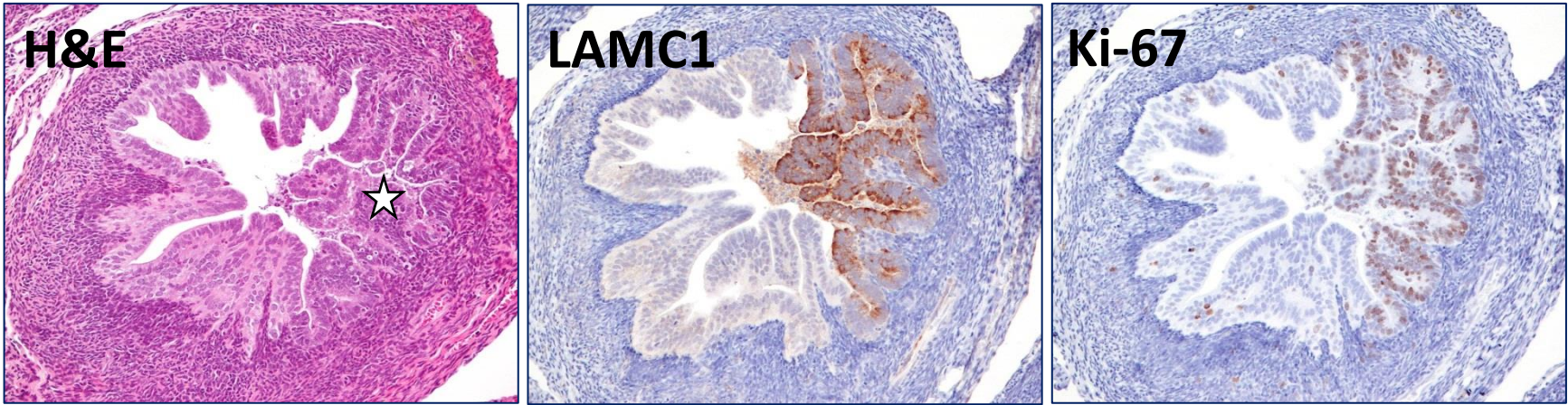
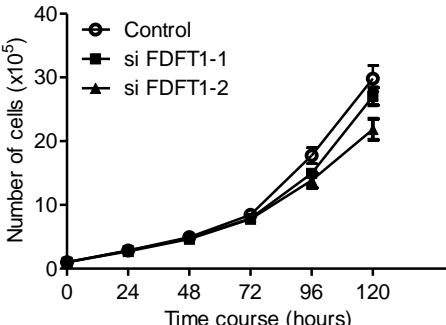
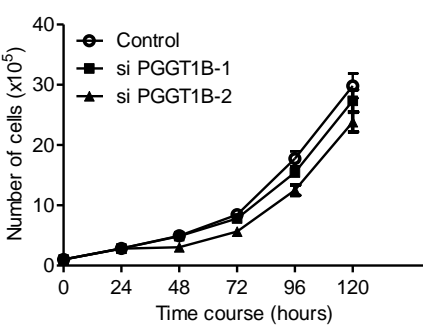
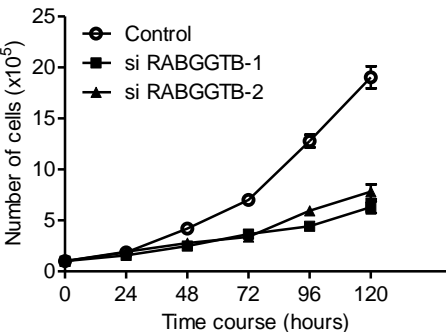
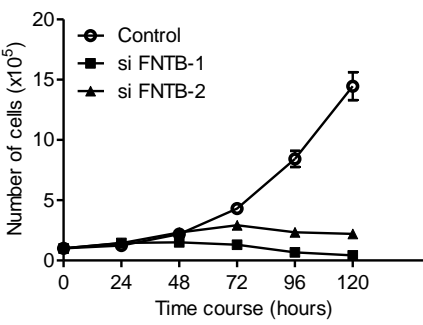
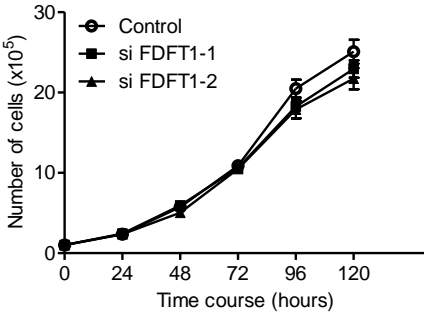
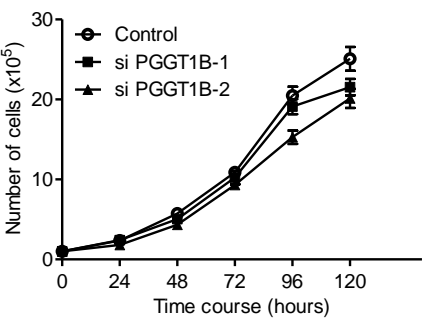
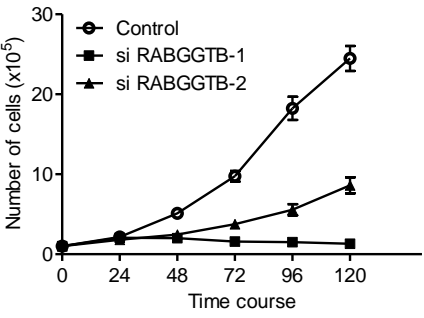
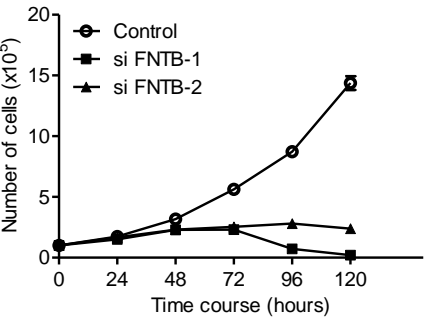


Figure 5

SKOV3



OVCAR5



Mevalonate Pathway Antagonist Inhibits Proliferation of Serous Tubal Intraepithelial Carcinoma in Mouse Models

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ABSTRACT

Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the mevalonate pathway for synthesis of cholesterol and other bioactive molecules. Statins are among the most frequently prescribed drugs because of their efficacy and low toxicity in treating hypercholesterolemia. Statins have been recently reported to inhibit proliferative activity in cancer cells, especially those with *TP53* mutations. Since *TP53* mutations occur in almost all cases of ovarian high-grade serous carcinoma, the most common and lethal type of ovarian cancer, many of which demonstrate up-regulation of mevalonate pathway genes, we determined if the statin, lovastatin, suppressed formation of the ovarian cancer precursor lesion, serous tubal intraepithelial carcinoma (STIC) in a mouse tumor model. Our results demonstrated that lovastatin administration significantly reduced the extent of STIC. Furthermore, using an ovarian cancer xenograft model, we observed that lovastatin treatment significantly inhibited tumor growth. Knockdown of the prenylation enzymes in the mevalonate pathway recapitulated the lovastatin-induced anti-proliferative phenotype. Transcriptome analysis demonstrated that lovastatin affects the expression of MCM replicative helicases and genes involved in the Rho/PLC signaling in addition to regulating the cholesterol biosynthesis genes, suggesting that statin has diverse effects on tumor cells through transcriptional effects in addition to its well-known role in directly inhibiting cholesterol synthesis. These results suggest that repurposing statin drugs for chemoprevention and treatment of ovarian cancer may provide a better strategy to prevent and manage this devastating disease.

INTRODUCTION

The incidence of epithelial ovarian cancer in the US has changed very little in the last 20 years; about 22,000 women will receive a new diagnosis this year. Because of the aggressiveness of the disease, once diagnosed, the overall 5-year survival rate is expected to be less than 50%. Part of the problem is that it is difficult to detect ovarian cancer at early stage and when diagnosed at late stages, there are limitations of effective treatments at this moment. Hence, it is critical to develop preventive strategy to reduce the risk of this dismal disease. Currently, for women who are BRCA mutation carriers, bilateral salpingo-oophorectomy (BSO) is the recommended surgical procedure to protect these women from developing ovarian cancer. In addition, oral contraceptives which reduce the frequency of ovulation, has been shown to be effective in reducing the incidence as well as mortality without significant adverse effects (PMID: 18294997). However, patients who undergo BSO procedures may suffer from post-surgery complications especially those symptoms associated with decreased hormone levels which include increased adiposity, cardiovascular disease, osteoporosis, and depression at relatively early ages (PMID: 19178933, PMID: 17617523, PMID: 25311333, PMID: 25342222, PMID: 19178933). Therefore, safe, and cost-effective chemopreventive strategies aimed at preventing or delaying the development of EOC are in urgent need.

One potential approach toward chemoprevention is to repurpose existing drugs that are safe and efficacious, and can be widely prescribed for exploiting its potency in cancer control and prevention. One such class of drugs are statins which target 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in the mevalonate pathway. The reasons to focus on statins are multifold. First, statins are one of the most frequently prescribed drugs to prevent treat hypercholesterolemia. Statins usage has been reported to be about 11% in the overall US population and as high as 44% in people above 65 years (PMID: 22812021). According to the Center of Disease Control, 25% of adults 45 years and over in the US were prescribed statins between 2005 and 2008 (CDC, US 2010, feb 16,2011). Second, statins have been found to be well-tolerated in patients, and side effects including alterations in liver function and muscle weakness or tenderness occur in only a small fraction of people (REF). Third, it has been recently reported that statins can inhibit proliferative activity of cancer cells, especially those with *TP53* mutations (REF). *TP53* mutations occur in virtually all ovarian high-grade serous carcinoma (HGSC) and in its precursor lesion, serous tubal intraepithelial carcinoma (STIC), suggesting that statin drugs maybe effective on HGSC and STIC. Fourth, epidemiologic studies have demonstrated reduced cancer mortality associated with statin use (@ Elmore, 2008 #5253;Lavie, 2013 #5260; Nielsen, 2012 #5282).

In this study, we determined anti-tumor effects of lovastatin, a lipophilic statin, in two animal models of ovarian cancer. The first model was a genetically engineered mouse model, mogp-Tag, in which the promoter of oviduct glycoprotein 1 (OVGP1) was used to drive expression of SV40 T antigen in gynecologic organs. These mice spontaneously develop STICs and ovarian/tubal carcinomas at a relatively young age. This transgenic mouse model displays a stepwise progression from normal tubal epithelium to invasive epithelial ovarian cancers, simulating the pathogenesis in humans. The second model was a xenograft tumor model in which human ovarian cancer cells are inoculated into immunocompromised mice. Using the transgenic mouse model, we determined the capacity of lovastatin, as a chemopreventive agent, to suppress formation of STICs. Using the xenograft model, we demonstrated the potency of lovastatin in delaying growth of ovarian tumors. We also explored the molecular mechanisms underlying the anti-tumor effects of lovastatin.

Results

Upregulation of Mevalonate Pathway Genes in Human Ovarian High-Grade Serous Carcinomas (HGSC)

In view of the epidemiologic evidence that ovarian cancer mortality is reduced in patients receiving statins, it is important to examine whether the mevalonate pathway was up-regulated

in ovarian cancer. We analyzed the expression levels of enzymes involved in the mevalonate pathway using the ovarian high-grade serous carcinoma (HGSC) dataset from TCGA. This dataset includes Affymetrix HT_HG-U133A microarray data from 564 primary HGSCs, 17 recurrent HGSCs, and 8 samples from normal fallopian tubes. Heatmap of gene expression and hierarchical clustering of all tumor samples was performed (Fig. 1). On one hand, we observed that probes in mevalonate pathway were consistently down-regulated in 7 out of 8 normal samples (Fig. 1). On the other hand, pathway expression in tumor samples formed three distinct clusters: tumor samples in cluster 1 (309 samples) and cluster 3 (52 samples) had significantly stronger expression in mevalonate pathway compared to 8 normal samples, while no significant change was observed for tumor samples in cluster 2 (220 samples). Table 1 gives the probe level p-values calculated by two-sample t-test, comparing gene expression in 8 normal samples versus 581 tumor samples. 13 out of 31 probes in this pathway had significant differential expression (p-value < 0.05) between tumor and normal samples. This expression pattern suggests that inhibition of the mevalonate pathway may have therapeutic potential for HGSCs.

A previous study demonstrated that missense mutations of TP53 upregulated expression of genes in the mevalonate pathway (PMID: 22265415). Hence, we performed analysis to determine if cluster 1 or cluster 3 tumor samples were associated with TP53 missense mutations. Mutation status was available in 305 of the 581 HGSC samples, among which 187 samples harbored missense mutation of TP53; the remaining cases harbored mutations that led to loss or truncation of TP53 protein including nonsense mutations, splice site mutations, and deletion or insertion mutations. We found that missense mutation status was not associated with any particular cluster as the three clusters exhibited similar % of tumors harboring TP53 missense mutation (Supplemental Table 1, Fisher's exact test).

Lovastatin Delays Progression of Spontaneous Gynecologic Neoplasms in a Transgenic Mouse Model

To determine if inhibition of the mevalonate pathway prevents tumor development, we employed a genetically engineered mouse model that expresses the SV40 large T antigen driven by the oviduct glycoprotein-1 (OVGP-1) promoter (mogp-TAg mice) (PMID: 12203826). Mogp-TAg mice consistently develop spontaneous serous tubal intraepithelial carcinoma, the precursor lesion of HGSC and uterine stromal sarcoma at 6-7 weeks of age. The mice were treated with lovastatin or control vehicle beginning at 3 weeks of age. The animals were euthanized at 8 weeks for endpoint study of tumor burden. The results demonstrated that treatment with lovastatin at either dose significantly reduced the tumor mass in the reproductive tract as evidenced by the organ weight (Fig. 2A). Serum levels of cholesterol and triglyceride were measured at endpoint, and the data showed significantly reduced cholesterol levels and marginally reduced triglyceride levels in mice treated with lovastatin compared to controls (Supplemental Fig. 1). To determine whether lovastatin inhibited the formation of STICs, we compared histopathology of fallopian tubes between lovastatin-treated and vehicle-treated mice. The data demonstrated that mice treated with lovastatin had fewer foci of STIC and a lesser extent of STIC in individual fallopian tubes than vehicle-treated mice (Fig. 2B). We then quantified STICs by immunohistochemistry on tissue sections using a STIC-associated marker, laminin C1 (PMID: 22892598). We found that the percentage of laminin C1- positive tubal epithelial cells in lovastatin treated mice was significantly reduced compared to the vehicle control group (Fig. 2C & 2D). Similarly, based on Ki-67 indices, the proliferative activity in STICs of the lovastatin-treated mice was significantly decreased compared to the control group (Fig. 2C, 2D).

The two doses of lovastatin employed in this study appeared to be safe in animals. There was no evidence of lethargy, weight loss, or other physical compromise. Necropsy was performed at the end point and histopathological examination of liver, spleen, kidney, heart, intestine, and brain did not reveal tissue damage in lovastatin-treated mice.

Lovastatin Inhibits Tumor Growth in a Human Ovarian Cancer Xenograft Model

The studies in the genetically engineered mouse model demonstrated the potency of lovastatin in suppressing spontaneously developing STICs and uterine stromal sarcoma. Next, we determined whether lovastatin exerted anti-tumor effects on established ovarian tumors in a xenograft model in immunocompromised mice. SKOV3-IP or OVCAR5 ovarian cancer cells were inoculated subcutaneously into athymic *nu/nu* mice. Beginning one week after tumor inoculation, lovastatin (12.5 mg/Kg) or vehicle control was administered by intraperitoneal injection twice a week for four weeks. All mice were evaluated for tumor growth twice a week until day 28 when animals were euthanized for endpoint study of tumor burden. Lovastatin administration significantly reduced the rate of increase in tumor sizes in both SKOV3-IP and OVCAR5 tumor xenografts (Fig. 3, left panels). Immunohistochemistry was performed on the excised tumors using antibodies to Ki-67 and phosphorylated histone 3 (a marker for dividing cells) to assess proliferative activity. The data showed that tumors from lovastatin-treated animals had significantly fewer proliferating cells than tumors from vehicle-treated animals (Fig. 3, middle and right panels).

The Effects of Lovastatin on Autophagy, Cellular Proliferation, and Apoptosis in Ovarian Cancer Cells

We next tested whether statin treatment affected apoptosis and autophagy in the tumor xenografts by Western blot analysis using antibodies against markers in both pathways. LC3A and LC3B are two isoforms of microtubule-associated protein 1 light chain, LC3, which undergo post-translational modification during autophagy. Cleavage of LC3 at the C-terminus yields cytosolic LC3-I. During autophagy, LC3-I is converted to LC3-II through lipidation by a ubiquitin-like system involving Atg7 and Atg3 that allows LC3 to become associated with autophagosomes (6-10). The conversion of LC3-I to the lower migrating form, LC3-II, was used as indicators of autophagy (11). We found that LC3A-II and LC3B-II were more abundant in OVCAR5 and SKOV3-IP tumor xenografts in the statin-treated group than in the vehicle-treated group (Fig. 4). Activation of apoptosis in OVCAR5 and SKOV3-IP tumor xenografts was determined by Western blot analysis using antibodies against cleaved caspase-3 and cleaved PARP1. We found a significant increase of both apoptosis markers in the SKOV3-IP xenografts but to a less extent in the OVCAR5 tumor xenografts (Fig. 4A).

In addition to tumor xenografts, we determined the short-term effects of lovastatin on SKOV3-IP and OVCAR5 cell cultures. Cells were incubated with lovastatin or vehicle control for 0, 6, 12, 24, 36, and 48 h and the expression levels of markers of autophagy and apoptosis were determined by Western blot. Autophagic activity was detected as early as 12 h after lovastatin incubation, while early apoptosis as detected by cleavage of caspase-3 and PARP1 was detected 36 h after statin exposure (Fig 4B). Next, we performed cell cycle analysis in lovastatin-treated SKOV3 and OVCAR5 cells using flow cytometry. Lovastatin treatment resulted in significant accumulation of ovarian cancer cells in G0/G1 phase in a dose-dependent manner (Fig. 4C); a concomitant decrease in the number of cells in G2/M phase was observed (Fig. 4C).

Lovastatin Affects Expression of Genes Involved in DNA Replication, Ras/Rho Signaling, and Cholesterol Biosynthesis

To elucidate the molecular mechanism leading to the observed anti-tumor effects of lovastatin, we performed global gene expression analysis using the Illumina Bead Array in lovastatin-treated ovarian cancer cells. In OVCAR5 1309 genes and in SKOV3 4128 genes were differentially expressed following lovastatin treatment (fold change > 1.7 and FDR < 0.05). Of these, 693 genes overlapped between OVCAR5 and SKOV3 ($p=1.495 \times 10^{-268}$, hypergeometric test). Ingenuity® Pathway Analysis (QIAGEN, Redwood City) demonstrated that the most

frequently involved canonical pathways included cell cycle control of DNA replication and phospholipase C (PLC) signaling (Fig. 5A; Table 2). In addition, several members of Rho and Ras small G protein families are within the PLC signaling pathway. We also performed gene set enrichment analysis (GSEA) to determine enrichment of KEGG functional pathways in our microarray data. The GSEA results were in agreement with the IPA pathway analysis with the gene set involving DNA replication ranked at the top of the list (Supplemental Table 2, Fig. 5B). Interestingly, we observed that several genes in the mevalonate pathway including HMGCS1 and HMGR were upregulated in cells treated with lovastatin, suggesting that tumor cells responded to mevalonate pathway blockage by transcriptionally upregulating genes in the same pathway to compensate for the reduced pools of pathway products. Similar regulation of enzymatic activity by transcription in response to metabolite levels in the same pathway is well-documented, and is conserved between yeast and mammals (PMID: 21801748). Furthermore, a significant fraction of genes in the glycolysis/gluconeogenesis pathway was upregulated in cells treated lovastatin (Fig. 5C, Supplemental Table 3)

To confirm expression changes induced by lovastatin treatment, we used qRT-PCR to assess mRNA expression of several members in the DNA replication and mevalonate biosynthesis pathways in tumor xenografts as well as in tumors derived from OVGP1 mice (Fig. 5D). The expression levels of MCM2-7 and MCM10, which encode minichromosome maintenance (MCM) proteins essential for initiation and elongation of DNA replication, were consistently down-regulated in lovastatin treated tumors (Fig. 5D). In contrast, the expression of HMGCS1 and HMGR which encode enzymes in the mevalonate pathway and PC, PCK1, PCK2, PKM2, HK1 and HK2, which are enzymes in the glycolysis pathway, were significantly up-regulated in lovastatin treated tumors compared to tumors derived from control treatment (Fig. 5D).

Protein Prenylation Mediates Anti-Proliferative Phenotype of Lovastatin

To determine if metabolites in the mevalonate pathway including cholesterol, CoQ10, GGPP, or FPP could rescue lovastatin-induced anti-proliferative effects in ovarian cancer cells, we cultured OVCAR5 and SKOV3 cells in the presence of each metabolite as well as lovastatin (10 μ M). The addition of GGPP significantly reverted the anti-proliferative ability of lovastatin (Fig. 6A & 6B), while GGPP alone did not affect proliferation (data not shown). In contrast, co-incubation of ovarian cancer cells with lovastatin and FPP, water-soluble cholesterol, or CoQ10 had no effect on lovastatin-induced anti-proliferative effects (Fig. 6A & 6B). These data suggest that the anti-proliferative effect of lovastatin is likely mediated by depletion of endogenous GGPP pools and is less likely to be related to cholesterol.

Since the above rescue assay indicated that geranylgeranylation subpathway was involved in the cytotoxic effect statin, we performed RNAi approach to further dissect key enzymes in this subpathway. The expression of geranylgeranyltransferases including PGGT1B and RABGGTB was down-regulated in SKOV3 and OVCAR5 cells by two different siRNAs targeting each enzyme. The knockdown efficiency was confirmed by qRT-PCR (Supplemental Fig. 2). Squalene synthase (FDFT1), a critical enzyme in the cholesterol synthesis subpathway, was also included as an experimental control. Knockdown of PGGT1B or RABGGTB significantly reduced proliferation, while knockdown of FDFT1 did not have a detectable effect (Fig. 6C & 6D).

Discussion

Although anticancer actions of statins in both tumor cell culture and xenograft models have been reported previously (PMID: 21688263), the chemopreventive potential of statins in spontaneous tumor mouse models has not been previously assessed. We report that lovastatin treatment prevents the formation of the ovarian cancer precursor, serous tubal intraepithelial

carcinoma (STIC) in the OVGP-1 Tag mouse model. We demonstrate that lovastatin also reduced tumor volume of pre-established ovarian tumor xenografts. As statin drugs have been widely prescribed to prevent cardiovascular disease and exhibit low toxicity, our results warrant investigation of statins to determine their clinical benefit in preventing ovarian cancer and in treating advanced stage ovarian cancer.

The anti-tumor effect of statins is probably through its pleiotropic actions. For example, statins have also been reported to modulate the tumor microenvironment to suppress immune surveillance, which provides permissive environment for tumor initiation and progression. In addition, this study identified geranylgeranylation is a critical molecular mechanism in the anti-proliferative activity of statins. Geranylgeranylation involves covalent addition of the GGPP lipid to a conserved motif on proteins and is an essential step in controlling protein localization. In members of the Rho/Rab or phospholipase superfamilies, geranylgeranylation specifies their localization to cellular membranes which is critical for signaling activation {Konstantinopoulos, 2007 #7769}. Rho GTPases are closely involved in cancer cell morphogenesis, motility, and migration. Rab GTPases controls membrane and vesicle trafficking. Phospholipases (PLC, PLD, and PLA) are essential mediators of intracellular signaling and regulate multiple cellular processes that can promote tumorigenesis. Given the importance of geranylgeranylation in regulating these signaling pathways, their inhibition is considered a promising target for cancer treatments (PMID: 23076158). Our knockdown study showing that enzymes involved in geranylgeranylation are critical for tumor cell growth further strengthens this view [40]. Thus, reducing the amount of GGPP by inhibiting the mevalonate pathway may compromise Rho/PLC signaling and suppress tumorigenesis.

The pleiotropic anti-tumor effects of statins were further supported by our transcriptome analysis, which showed that lovastatin-regulated genes participate in a wide spectrum of functional pathways including DNA replication, Rho/PLC signaling, and glycolysis in addition to cholesterol biosynthesis. Therefore, the diverse functions of statins may contribute to their anti-tumor potential in addition to their well-known role in inhibiting cholesterol synthesis. The observed upregulation of mRNA of mevalonate pathway genes such as HMG-CoA reductase is not surprising because the negative feedback regulation of transcription in response to the inhibition of HMG-CoA reductase is well-documented (PMID: 1967820). Another potential anti-tumor mechanism of statins stems from our results demonstrating that several genes involved in DNA replication were downregulated by statins in tumor cells. Most notably, mRNA levels of 7 MCM genes were significantly decreased by lovastatin treatment. MCM proteins are known to form replicative helicase complexes, which play a pivotal role not only in DNA initiation and elongation but also in DNA damage response, transcriptional regulation, and modulating chromatin structure {Forsburg, 2004 #7773} {Bell, 2013 #7774}. Thus, statins may directly or indirectly silence the expression of MCM genes, leading to cell cycle arrest and accumulation of DNA damage.

In our mouse models, we observed that lovastatin effectively inhibited tumor growth at either the precursor stage or in established xenografted tumors but had no noticeable effect on normal tissues. The reasons for the differential effects of statins on normal and neoplastic cells are unclear, but several possibilities can be postulated. First, ovarian tumor cells, as compared to normal cells, may have become more dependent on the mevalonate pathway for sustaining cellular survival and growth. In fact, mevalonate pathway activity has been found to be enhanced in many malignancies, including myeloid leukemia, lung, and breast cancers. Additionally, expression levels of HMG-CoA reductase were increased in neoplastic tissues (PMID: 24777857, PMID: 24708688). Supporting this view, our result demonstrated that more than half of ovarian high-grade serous carcinomas in the TCGA dataset overexpressed genes

belonging to the mevalonate pathway, likely because tumor cells, in contrast to most normal cells, acquire *de novo* synthesis capability to produce excessive quantities of mevalonate pathway products for cellular survival. Second, the transcriptional network has been re-programmed in cancer cells, allowing the mevalonate pathway to participate in transcriptional activities including upregulation of MCM genes that collectively promote tumor progression. Statins suppress these tumor cell activities and, subsequently, tumor growth, although it remains to be determined precisely how statins affect transcriptional activities.

Our findings provide critical pre-clinical data, which warrant future translational studies aimed at repurposing statins for prevention and treatment of ovarian cancer. STIC has been thought to be the precursor lesion in most ovarian high-grade serous carcinomas (reviewed in {Kuhn, 2012 #7088}), and therapeutic intervention to surgically remove fallopian tubes that may harbor STICs has been advocated for reducing ovarian cancer risk, especially for women with predisposing BRCA1/BRCA2 mutations. As an alternative to such an invasive procedure, it will be of interest to determine if risk of ovarian cancer is reduced by statin treatment. For example, prospective randomized controlled studies can be performed to confirm the benefits of statin in reducing the risk of ovarian cancer and therapeutic clinical trials will be needed to test if statins can reduce ovarian cancer mortality. Repurposing statin drugs thus may have a substantial impact on managing women with increased ovarian cancer risk as well as ovarian cancer patients.

Figure Legends

Fig. 1. Expression of mevalonate pathway genes in ovarian cancer. Heatmap demonstrates expression of genes in the mevalonate pathway in ovarian cancer and normal fallopian tube based on TCGA dataset. Expression of each probe was standardized using log scale where red color represents upregulation and green color represents downregulation. Each row represents one sample and each column corresponds to one probe in the mevalonate pathway. Normal samples in general have low expression of genes in the mevalonate pathway. Expression of tumor samples formed three clusters. Samples in cluster 1 and 3 had significant upregulation of mevalonate pathway.

Fig. 2. Lovastatin prevents tumor growth in OVGP1-SV40 transgenic mice.

A. Daily administration of lovastatin (50 mg/Kg or 100 mg/Kg) was initiated when the OVGP-1 mice were 3 weeks old, and the end point necropsy was performed when the mice were 8 weeks old. Right: Representative images of gynecological tracts from OVGP1 mice at the end point. Left: Weight of gynecological tracts taken from OVGP1 mice treated with lovastatin or control vehicle (n= 10 for each group; ***p< 0.001). **B.** Representative section of fallopian tube from OVGP1 mice treated with lovastatin or with vehicle control. **C.** Summary of LAMC-1 and Ki-67 staining. Bar graphs depict percent of LAMC-1-positive or Ki-67-positive epithelial cells among total FT epithelial cells per fallopian tube section. In each experiential group, data were collected from 10 representative sections from each of 10 mice; **p<0.01. **D.** Representative images of H&E, LAMC-1, and Ki-67 staining on tissue sections from fallopian tubes of OVGP1 mice.

Fig. 3. Lovastatin suppresses growth of human ovarian tumor xenografts. SKOV3-IP (A) or OVCAR5 (B) cells were injected subcutaneously into athymic *nu/nu* mice. One week after tumor inoculation, mice received i.p. treatment of lovastatin (12.5 mg/kg) twice a week until the termination of the study. Tumor volume was measured by a caliper twice per week. The mean tumor volumes are plotted \pm SD. Middle: Bar graphs depict percent of Ki-67-positive cells per high power field (400 X). Five high power (400X) fields were screened per tumor, and in total, 25 high power fields were included for each experimental group of 5 mice. Right: Bar graphs depict average number of phospho-Histone H3-positive cells per high power field (400X). Ten

high power (400X) fields were collected from each tumor (section), and in total, 50 high power fields were included for each group. Middle: Bar graphs depict percent Ki-67-positive cells in control and statin-treated tumors. Data were collected from five high power (400X) fields for each group, and are presented as mean \pm SD. Right: Bar graphs depict average number of phospho-Histone H3-positive cells in control and statin-treated tumors detected in ten high power (400X) fields. Data represent averages of five different tumors.

Fig. 4. Lovastatin induces autophagy and cell cycle arrest in ovarian cancer cells. **A.** SKOV3-IP and OVCAR5 xenograft tumors from control and lovastatin-treated mice were excised, lysed, and analyzed by Western blotting using antibodies against cleaved caspase-3 and cleaved PARP1 to detect autophagy by LC3-II conversion and apoptosis by cleavage of poly(ADP-ribose) polymerase (PARP) and caspases-3. Blots were stripped and re-probed with GAPDH antibody to verify equal protein loading. Each lane represents a different xenograft tumor sample. **B.** SKOV3 and OVCAR5 cell cultures were incubated with lovastatin or vehicle alone for various times and were harvested for Western blot analysis using antibodies against cleaved caspase-3 and cleaved PARP1 to detect autophagy and apoptosis. **C.** SKOV3 and OVCAR5 cells were treated with 0, 1, 10, or 100 μ M lovastatin for 48 hrs. Cell cycle was measured by flow cytometry using propidium iodide (PI) staining. The percentage of cells in G0/G1, S, and G2/M phases are depicted.

Fig. 5. Genome-wide expression profiling of lovastatin-regulated genes using *in vitro* and *in vivo* tumor models. **A.** Ingenuity Pathway Analysis of statin-regulated genes. **B.** The lovastatin-regulated genes in SKOV3 and OVCAR5 tumor cells were then compared with the KEGG functional pathways to evaluate gene set enrichment. Shown is the top down-regulated gene set, DNA replication. **C.** Expression levels of genes in glycolysis/gluconeogenesis pathway. Red circle: upregulated by lovastatin; blue circle: down-regulated by lovastatin; circles with black outlines indicate the data are statistically significant. The relative expression values of each data circle can be found in Supplemental Table. **D.** qRT-PCR analysis of expression of genes in DNA replication and sterol biosynthesis pathways in SKOV3-IP and OVCAR5 tumor xenografts and in spontaneous tumors derived from OVGP1 mice. Normalized expression values derived from three replicated wells for each gene; green pseudocolor coding represents downregulation and red coding represents upregulation.

Fig. 6 Exogenous GGPP rescues the anti-proliferative effect of lovastatin. SKOV3 (**A**) and OVCAR5 (**B**) cells were incubated with GGPP (25 μ M), FPP (25 μ M), water-soluble cholesterol (400 μ g/ml), or CoQ10 (25 μ M) alone or were co-incubated with lovastatin (10 μ M). Since GGPP and FPP alone did not affect proliferation, their plots were omitted to simplify the presentation. Proliferation was evaluated by cell-titer blue assay. Data are presented as the mean \pm SD (N = 3). **C & D.** siRNAs against key enzymes in the geranylgeranylation and squalene synthesis pathways, including PGGT1B, RABGGTB, and FDFT1, were transfected into ovarian cancer cell lines SKOV3 (**C**) and OVCAR5 (**D**) by Lipofectamine 2000. Proliferation was evaluated from day 1 to day 5 using cell-titer blue assay.

Supplemental Fig. 1. Body weight and plasma levels of cholesterol and triglyceride in OVGP1, SKOV3-IP, and OVCAR5 mouse tumor models. Lovastatin significantly reduced cholesterol in all three mouse models but did not affect mouse body weight. Lovastatin elicited mixed responses in plasma triglyceride levels.

Supplemental Fig. 2. Real-time qRT-PCR confirms knockdown efficiency of each siRNA. The knockdown efficiency of each siRNA for the designated enzyme in the mevalonate pathway

was analyzed by qRT-PCR. Scrambled siRNA was used as a negative control. All values were normalized to expression of β -actin (ACTB), a housekeeping gene.

Material and Methods

TCGA Analysis

Gene expression data of ovarian samples (Affymetrix HT_HG-U133A) were downloaded from The Cancer Genome Atlas (TCGA) data portal, which included 564 primary solid tumor samples, 17 recurrent solid tumor samples, and 8 samples from solid normal tissue. Raw .CEL files of the 589 samples were normalized using Probe Logarithmic Intensity Error (PLIER) algorithm from Affymetrix Expression Console. We used the ComBat algorithm [1] from GenePattern (<http://www.broadinstitute.org/cancer/software/genepattern/>) to correct for potential batch effects in the data. Data after batch correction included 22,215 probes, whose intensity values were in log-scale. Batch adjusted gene expression data were further standardized (scaled to normal distribution with mean 0 and standard deviation 1) across all 589 samples for each probe.

Johnson WE, Li C, Rabinovic A: **Adjusting batch effects in microarray expression data using empirical Bayes methods.** *Biostatistics* 2007, **8**(1):118-127.

Animal Studies

The generation of the mogp-TAg transgenic mouse has been described (12). Mice were housed and handled according to the approved protocol by the Johns Hopkins University Animal Care and Use Committee. The genotype of the OVGP1 transgene was confirmed by tail DNA extraction and polymerase chain reaction (PCR). PCR was performed using the following conditions: denaturation at 94°C for 30 sec, followed by 30 cycles at 94°C for 15 sec, 55°C for 30 sec, 68°C for 45 sec, and final extension at 68°C for 5 min. The primer sequences were: Forward -GAAAATGGAAGATGGAGTAAA-, Reverse-AATAGCAAAGCAAGCAAGAGT-. OVGP1 mice were treated with 50 mg/kg lovastatin diluted in 0.5% methylcellulose, by gastric intubation using disposable feeding tubes beginning at 3 weeks of age until euthanasia at 8 weeks. Reproductive tracts were removed, weighed, formalin-fixed, and embedded in paraffin. Since tumor cells occupy approximately 50% of total tissue mass in untreated mice, tissue weight was used as an indicator of tumor burden.

To test the therapeutic potential of lovastatin, we used mouse xenograft tumor models. Human ovarian cancer cells, SKOV3-IP and OVCAR5 cells (5×10^6) were injected subcutaneously into the left flank of 6-week-old female mice. The mice were randomly assigned to treatment or control groups; beginning one week after tumor cell inoculation, lovastatin (12.5 mg/kg per injection) or vehicle control was injected intraperitoneally twice weekly (PMID: 21816418). Tumor diameters were measured twice per week using a caliper. Tumor volume (V) was calculated using the formula: $V = A \times B^2/2$ (where A = axial diameter; B = rotational diameter). Excised tumors were either homogenized and RNA was extracted using an RNeasy Mini Kit (Qiagen) or were fixed overnight in neutral-buffered formalin and embedded in paraffin blocks.

Blood from mouse tails was collected into microfuge tubes containing EDTA. The tube was centrifuged at 3,000 rpm for 20 min, and the plasma was submitted to the clinical chemistry laboratory at Johns Hopkins Hospital for analysis of cholesterol and triglyceride contents.

Cell culture

The cell lines used in this study, including SKOV3, OVCAR5, OVCAR3, were purchased from American Type Culture Collection (ATCC). SKOV3-IP is a derivative line of SKOV3 after three passages in athymic *nu/nu* mice and is potently tumorigenic. All cell lines were cultured at 37°C, 5% CO₂ in RPMI-1640 supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 U/mL).

Western Blotting Analysis

Tumor tissues or cells were homogenized in lysis buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1% NP40) with Halt™ Protease Inhibitor Cocktail (1861278, Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration in tissue or cell lysate was determined using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard. An aliquot of protein

lysate (30 µg) was separated by SDS-PAGE, and Western blot was performed using standard procedures. Blots were developed using Amersham ECL Western Blotting Detection Reagents kit (GE Healthcare UK Ltd, Buckinghamshire, UK). Primary antibodies used in this study: LC3A (#4599; Cell Signaling Technology, Inc, Danvers, MA); LC3B (#3868; Cell Signaling Technology); p21 (sc-6246; Santa Cruz Biotechnology); Cleaved Caspase-3 (#9664; Cell Signaling Technology); PARP (#5625; Cell Signaling Technology); and GAPDH (#5174; Cell Signaling Technology).

Immunohistochemistry

Paraffin-embedded tissue sections (4 µm) were deparaffinized in xylene and rehydrated in graded alcohols. Heat induced epitope retrieval was then performed by incubation in Trilogy™ (Cell Marque, Austin, TX, catalog # CMX833) in a steamer. Endogenous peroxidase activity was blocked in a bath of H₂O₂ solution for 15 min, following by blocking with Dako Antibody Diluent (DAKO) at room temperature for 30 min. Sections were incubated with antibody diluted in Dako Antibody diluent at 4°C overnight. Positive reactions were detected by applying EnVision™+HRP polymer (Dako, Carpinteria, CA, catalog # K4003) for 30 min, followed by incubation in DAB substrate for 5 min (Dako Liquid DAB+, Dako, Carpinteria, CA, catalog #K3468). The slides were then counterstained with hematoxylin to visualize the cell nuclei (Sigma-Aldrich Corporation, St Louis, MO, catalog # HHS32). Antibodies used in this study were: human Ki-67 (cat # M7240, DAKO), mouse Ki-67 (cat # 12202 Cell Signaling Technology), LAMC1 (cat # HPA001908, Sigma-Aldrich), phospho-Histone H3 (Ser10) antibody (cat # 9701, Cell Signaling Technology), and LC3B (cat # 3868, Cell Signaling).

Quantification of immunohistochemical staining

The Ki-67 or LAMC1 positivity in fallopian tubal epithelium of OVGP-1 mouse was quantitated as the percentage of positively stained cells. At least 3,200 tubal epithelial cells were counted in each sample.

The proliferative index in xenografts were quantitated as the percentage of Ki-67 positively stained epithelial cells. The total number of epithelial cells and the number of positively stained epithelial cells were counted in each microscopic field (magnification, ×40; Nikon Orthoplan microscope); at least 10 random fields (greater than 2500 tubal epithelial cells) per experimental group were scored by two independent observers who were blinded to the treatment group. Differences in counts between the observers were <10%.

Reference

- 1) Rosenfeldt MT, Nixon C, Liu E, et al. Analysis of macroautophagy by immunohistochemistry. *Autophagy* 2012; 8: 963–969
- 2) Kashima J, Shintani-Ishida K, Nakajima M, et al. Immunohistochemical study of the autophagy marker microtubule-associated protein 1 light chain 3 in normal and steatotic human livers. *Hepato Res* 2013

Microarray Analysis

Quality and quantity of total RNA was determined using Agilent 2100 Bioanalyzer and a Nanodrop spectrophotometer, respectively. cRNA was synthesized using an Illumina RNA amplification kit (Ambion) starting from 500 ng total RNA and following the procedure suggested by the manufacturer. BeadChip hybridization, washing, and staining were performed according to the manufacturer's instructions. Arrays were scanned on an Illumina BeadStation 500. BeadChip array data quality control was done using Illumina BeadStudio software. Probe average intensity signal was calculated with BeadStudio without background correction. Empirical Bayes method (R package limma) was applied to assess the differential expression between DMSO- and Statin-treated cells. Differentially expressed probes were defined as having fold change greater than 1.7 and adjusted $p < 0.05$ (false discovery rate). Probe level data were summarized to gene level data by choosing the probe with the smallest p value for each gene.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed on gene expression microarray data using GSEA desktop application v2.0.14 (<http://www.broad.mit.edu/gsea/>) and KEGG gene sets from the Molecular Signature Database (MSigDB) version 4.0. After ranking genes according to log2 ratio of expression (control/statin), GSEA calculated enrichment scores and derived significance values by 2,000 permutations of the gene-set.

Quantitative RT-PCR Analysis

RNA was isolated using the RNeasy kit from Qiagen. Total cellular RNA (1 µg) was reverse transcribed into cDNA using an iScript cDNA kit (Bio-Rad). Real-time reverse transcription–PCR (RT-PCR) was performed using the CFX96 Real Time PCR Detection System (Bio-Rad) using SYBR Green I detection (Invitrogen). Primer sequences are listed in Supplemental Table 3. Relative quantitation of mRNA levels was plotted as fold increase compared with untreated samples. Actin B expression was used for normalization. ΔCt values (target gene Ct minus Actin B Ct) for each triplicate sample were averaged, and $\Delta\Delta\text{Ct}$ was calculated as the difference between statin treatment versus vehicle control in the same cell line. mRNA expression was determined from the formula: $2^{-\Delta\Delta\text{Ct}}$.

Table 1. Comparison of expression level of mevalonate pathway genes between HGSC and normal ovaries.

Probe-set	Gene Symbol	p-value*
209608_s_at	ACAT2	0.081314
202314_at	CYP51A1	0.031061
216607_s_at	CYP51A1	0.008453
200862_at	DHCR24	0.541522
201790_s_at	DHCR7	1.41E-05
201791_s_at	DHCR7	4.25E-06
208647_at	FDFT1	0.222953
210950_s_at	FDFT1	0.135892
201275_at	FDPS	0.016085
217344_at	FDPS	0.001449
202539_s_at	HMGCR	0.215722
202540_s_at	HMGCR	0.103099
205822_s_at	HMGCS1	0.035183
221750_at	HMGCS1	0.000747
204615_x_at	IDI1	0.253637
208881_x_at	IDI1	0.258267
202245_at	LSS	0.004292
211018_at	LSS	0.038814
211019_s_at	LSS	0.114369
203027_s_at	MVD	0.526764
204056_s_at	MVK	0.180087

215649_s_at	MVK	0.324366
36907_at	MVK	0.186977
209279_s_at	NSDHL	0.363313
215093_at	NSDHL	0.21174
203515_s_at	PMVK	0.72163
209146_at	SC4MOL	0.84955
209218_at	SQLE	0.002707
213562_s_at	SQLE	0.109668
213577_at	SQLE	0.002783
210130_s_at	TM7SF2	0.027816

*P-value was determined by two-sample t-test.

Table 2. IPA pathways regulated by lovastatin in ovarian cancer cells.

Ingenuity Canonical Pathways	-log(p-value)	Ratio	Molecules
Cell Cycle Control of Chromosomal Replication	5.38E+00	2.35E-01	MCM5,MCM3,MCM6,MCM2,CDT1,CDC6,ORC6,MCM4
Phospholipase C Signaling	3.23E+00	7.17E-02	ARHGEF4,PLD3,RRAS,PLA2G4C,MEF2A,ITGA5,CREB5,HMOX1,PLCB4,RHOB,AHNAK,RHOA,ARHGEF6,LAT,ITPR3,ARHGEF2,ARHGEF3,RHOF,RALGDS
Role of Tissue Factor in Cancer	3.21E+00	9.23E-02	VEGFA,IL8,CTGF,ARRB1,RRAS,ITGA6,IL1B,CSF2,CYR61,ITGB5,MMP1,ITGB3
Bile Acid Biosynthesis, Neutral Pathway	2.98E+00	6.90E-02	AKR1C1/AKR1C2,AKR1C3,AKR1C4,HSD3B7
VDR/RXR Activation	2.66E+00	1.02E-01	IGFBP6,SPP1,MXD1,CDKN1A,HES1,CEBPB,THBD,CSF2,NCOA3
Glioma Invasiveness Signaling	2.30E+00	1.06E-01	RHOB,RRAS,RHOA,PLAU,RHOF,ITGB5,ITGB3
Methylglyoxal Degradation III	2.17E+00	1.30E-01	AKR1C1/AKR1C2,AKR1C3,AKR1C4
Dopamine Degradation	2.16E+00	1.05E-01	ALDH1B1,SULT1A1,SULT1A3/SULT1A4,SMOX
Androgen Biosynthesis	2.06E+00	1.15E-01	AKR1C3,AKR1C4,HSD3B7
Reelin Signaling in Neurons	2.05E+00	9.41E-02	ARHGEF4,MAPT,ARHGEF6,ITGA6,ITGA5,ARHGEF2,ARHGEF3,ITGB3
IL-17A Signaling in Fibroblasts	2.04E+00	1.25E-01	TRAF3IP2,NFKBIA,LCN2,CEBPB,MMP1
Estrogen-mediated S-phase Entry	1.95E+00	1.43E-01	CDKN1A,E2F2,SKP2,CDC25A

Supplemental Table 1. Distribution of different types of TP53 mutation among three EOC clusters.

	Missense mutation	Other mutation	Not available*	Total	Missense/Total	Fisher's exact test [#]
Cluster1	19	9	24	52	0.67	0.5439
Cluster2	69	45	106	220	0.6053	0.9033
Cluster3	99	64	146	309	0.6074	0.9062

*mutation information unavailable.

[#] Fisher's exact test was used to test whether any of the clusters is associated with TP53 missense mutation. Data presented are p-values based on two-tailed analysis.

Supplemental Table 2. GSEA Enrichment of KEGG functional pathways in lovastatin-regulated genes

	NAME	NOM p-val	FDR q-val
SKOV3 Down-regulated by statin	KEGG_DNA_REPLICATION	0.000	0.000
	KEGG_HOMOLOGOUS_RECOMBINATION	0.001	0.002
	KEGG_MISMATCH_REPAIR	0.000	0.003
	KEGG_BETA_ALANINE_METABOLISM	0.002	0.002
	KEGG_NUCLEOTIDE_EXCISION_REPAIR	0.000	0.014
	KEGG_CELL_CYCLE	0.000	0.012
	KEGG_RNA_POLYMERASE	0.000	0.011
	KEGG_BASE_EXCISION_REPAIR	0.000	0.027
	KEGG_SPLICEOSOME	0.000	0.024
	KEGG_BUTANOATE_METABOLISM	0.024	0.066
	KEGG_LYSINE_DEGRADATION	0.004	0.075
	KEGG_PROGESTERONE_MEDIATED_OOCYT	0.001	0.083
	KEGG_PROTEASOME	0.003	0.080
	KEGG_PYRIMIDINE_METABOLISM	0.000	0.076
	KEGG_RIBOSOME	0.000	0.074
	KEGG_PENTOSE_AND_GLUCURONATE_INT	0.133	0.093
	KEGG_RNA_DEGRADATION	0.006	0.091
	KEGG_ONE_CARBON_POOL_BY_FOLATE	0.147	0.091
SKOV3 Up-regulated by statin	KEGG_TYPE_I_DIABETES_MELLITUS	0.000	0.006
	KEGG_GRAFT_VERSUS_HOST_DISEASE	0.001	0.012
	KEGG_ALLOGRAFT_REJECTION	0.000	0.013
	KEGG_AUTOIMMUNE_THYROID_DISEASE	0.002	0.036
	KEGG_GLYCOSAMINOGLYCAN_DEGRADAT	0.013	0.037
	KEGG_STEROID_BIOSYNTHESIS	0.032	0.054
	KEGG_COMPLEMENT_AND_COAGULATION	0.003	0.047
	KEGG_PRION_DISEASES	0.006	0.055
	KEGG_SNARE_INTERACTIONS_IN_VESICUL	0.001	0.075
	KEGG_LEISHMANIA_INFECTION	0.001	0.071
	KEGG_PPAR_SIGNALING_PATHWAY	0.000	0.087
OVCAR5 Down-regulated by statin	KEGG_DNA_REPLICATION	0.000	0.000
	KEGG_MISMATCH_REPAIR	0.000	0.003
	KEGG_NUCLEOTIDE_EXCISION_REPAIR	0.000	0.015
	KEGG_RNA_POLYMERASE	0.000	0.032
	KEGG_HOMOLOGOUS_RECOMBINATION	0.007	0.037
	KEGG_SPLICEOSOME	0.000	0.065
	KEGG_PRIMARY_IMMUNODEFICIENCY	0.108	0.074
	KEGG_PYRIMIDINE_METABOLISM	0.000	0.086
OVCAR5 Up-regulated by statin	KEGG_STEROID_HORMONE_BIOSYNTHESIS	0.000	0.007
	KEGG_METABOLISM_OF_XENOBIOTICS_BY	0.000	0.013